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1 Control of protein synthesis, and screening method for
2 agents.

3

4 The present invention relates to the control of
5 glycogen metabolism and protein synthesis, in
6 particular through the use of insulin.

7

8 Many people with diabetes have normal levels of insulin
9 in their blood, but the insulin fails to stimulate
10 muscle cells and fat cells in the normal way (type II
11 diabetes). Currently it is believed that there is a
12 breakdown in the mechanism through which insulin
13 signals to the muscle and fat cells.

14

15 The enzyme glycogen synthase kinase-3 (GSK3) (Embi et
16 al., 1980) is implicated in the regulation of several
17 physiological processes, including the control of
18 glycogen (Parker et al., 1983) and protein (Welsh et
19 al., 1993) synthesis by insulin, modulation of the
20 transcription factors AP-1 and CREB (Nikolaki et al, de
21 Groot et al., 1993 and Fiol et al 1994), the
22 specification of cell fate in *Drosophila* (Siegfried et
23 al., 1992) and dorsoventral patterning in *Xenopus*
24 embryos (He et al., 1995). GSK3 is inhibited by serine

1 phosphorylation in response to insulin or growth
2 factors (Welsh et al., 1993, Hughes et al., 1994, Cross
3 et al., 1994 and Saito et al., 1994) and *in vitro* by
4 either MAP kinase-activated protein (MAPKAP) kinase-1
5 (also known as p90^{rk}) or P70 ribosomal S6 kinase (p70^{S6k})
6 (Sutherland et al., 1993 and Sutherland et al., 1994).
7

8 We have now found, however, that agents which prevent
9 the activation of both MAPKAP kinase-1 and p70^{S6k} by
10 insulin *in vivo* do not block the phosphorylation and
11 inhibition of GSK3. Another insulin-stimulated protein
12 kinase inactivates GSK3 under these conditions, and we
13 demonstrate that it is the product of the proto-
14 oncogene Akt (also known as RAC or PKB; herein referred
15 to as "PKB").
16

17 GSK3 is inhibited in response to insulin with a half
18 time of two min, slightly slower than the half time for
19 activation of PKB α (one min). Inhibition of GSK3 by
20 insulin results in its phosphorylation at the same
21 serine residue (serine 21) which is targeted by PKB α in
22 *vitro*. Like the activation of PKB α , the inhibition of
23 GSK3 by insulin is prevented by phosphatidyl inositol
24 (PI-3) kinase inhibitors wortmannin and LY 294002. The
25 inhibition of GSK3 is likely to contribute to the
26 increase in the rate of glycogen synthesis (Cross et
27 al., 1994) and translation of certain mRNAs by insulin
28 (Welsh et al., 1994).
29

30 Two isoforms of PKB, termed PKB α (Coffer & Woodgett,
31 1991), PKB β (Cheng et al., 1992) and PKB γ (Konishi et
32 al., 1995) have been identified and characterised.
33 PKB β , also known as RAC β and Akt-2, is over-expressed
34 in a significant number of ovarian (Cheng et al., 1992)
35 and pancreatic (Cheng et al., 1996) cancers and is
36 over-expressed in the breast cancer epithelial cell

1 line MCF7. PKB is composed of an N-terminal pleckstrin
2 homology (PH) domain, followed by a catalytic domain
3 and a short C-terminal tail. The catalytic domain is
4 most similar to cyclic AMP-dependent protein kinase
5 (PKA, 65% similarity) and to protein kinase C (PKC, 75%
6 similarity) findings that gave rise to two of its
7 names, namely PKB (i.e. between PKA and PKC) and RAC
8 (Related to A and C kinase).

9
10 Many growth factors trigger the activation of
11 phosphatidylinositol (PI) 3-kinase, the enzyme which
12 converts PI 4,5 bisphosphate (PIP2) to the putative
13 second messenger PI 3,4,5 trisphosphate (PIP3), and PKB
14 lies downstream of PI 3-kinase (Franke et al., 1995).
15 PKB α is converted from an inactive to an active form
16 with a half time of about one minute when cells are
17 stimulated with PDGF (Franke et al., 1995), EGF or
18 basic FGF (Burgering & Coffey, 1995) or insulin (Cross
19 et al., 1995 and Kohn et al., 1995) or perpervanadate
20 (Andjelkovic et al., 1996). Activation of PKB by
21 insulin or growth factors is prevented if the cells are
22 preincubated with inhibitors of PI 3-kinase (wortmannin
23 or LY 294002) or by overexpression of a dominant
24 negative mutant of PI 3-kinase (Burgering & Coffey
25 1995). Mutation of the tyrosine residues in the PDGF
26 receptor that when phosphorylated bind to PI 3-kinase
27 also prevent the activation of PKB α (Burgering &
28 Coffey, 1995 and Franke et al., 1995).

29
30 The present invention thus provides the use of PKB, its
31 analogues, isoforms, inhibitors, activators and/or the
32 functional equivalents thereof to regulate glycogen
33 metabolism and/or protein synthesis, in particular in
34 disease states where glycogen metabolism and/or protein
35 synthesis exhibits abnormality, for example in the
36 treatment of type II diabetes; also in the treatment of

1 cancer, such as ovarian, breast and pancreatic cancer.
2 A composition comprising such agents is also covered by
3 the present invention, and the use of such a
4 composition for treatment of disease states where
5 glycogen metabolism and/or protein synthesis exhibit
6 abnormality.

7

8 The present invention also provides a novel peptide
9 comprising the amino acid sequence Arg-Xaa-Arg-Yaa-Zaa-
10 Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa
11 are any amino acid (preferably not glycine), and Hyd is
12 a large hydrophobic residue such as Phe or Leu, or a
13 functional equivalent thereof. Represented in single
14 letter code, a suitable peptide would be RXXR'X'S/TF/L,
15 where X' can be any amino acid, but is preferably not
16 glycine; glycine can in fact be used, but other amino
17 acids are preferred. Typical peptides include
18 GRPRTSSFAEG, RPRAATC or functional equivalents thereof.
19 The peptide is a substrate for measuring PKB activity.

20

21 The invention also provides a method for screening for
22 substances which inhibit the activation of PKB in vivo
23 by preventing its interaction with PIP3 or PI3,4-bisP.

24

25 Thus the invention also provides a method of
26 determining the ability of a substance to affect the
27 activity or activation of PKB, the method comprising
28 exposing the substance to PKB and phosphatidyl inositol
29 polyphosphate (ie PIP3, PI3,4-bisP etc) and determining
30 the interaction between PKB and the phosphatidyl
31 inositol polyphosphate. The interaction between PKB
32 and the phosphatidyl inositol polyphosphate can
33 conveniently be measured by assessing the
34 phosphorylation state of PKB (preferably at T308 and/or
35 S473), eg by measuring transfer of radiolabelled ³²P
36 from the PIP3 (for example) to the PKB and/or by SDS-

1 PAGE.

2

3 The method of the invention can also be used for
4 identifying activators or inhibitors of GSK3, such a
5 method can comprise exposing the substance to be tested
6 to GSK3, and (optionally) a source of phosphorylation,
7 and determining the state of activation of GSK3
8 (optionally by determining the state of its
9 phosphorylation. This aspect of the invention can be
10 useful for determining the suitability of a test
11 substance for use in combatting diabetes, cancer, or
12 any disorder which involves irregularity of protein
13 synthesis or glycogen metabolism.

14

15 The invention also provides a method for screening for
16 inhibitors or activators of enzymes that catalyse the
17 phosphorylation of PKB, the method comprising exposing
18 the substance to be tested to

19 - one or more enzymes upstream of PKB;

20 - PKB; and (optionally)

21 - nucleoside triphosphate

22 and determining whether (and optionally to what extent)
23 the PKB has been phosphorylated on T308 and/or S473.

24

25 Also provided is a method of identifying agents able to
26 influence the activity of GSK3, said method comprising:

27

28 a. exposing a test substance to a substrate of GSK3;

29

30 b. detecting whether (and, optionally, to what
31 extent) said peptide has been phosphorylated.

32

33 The test substance may be an analogue, isoform,
34 inhibitor, or activator of PKB, and the above method
35 may be modified to identify those agents which
36 stimulate or inhibit PKB itself. Thus such a method

1 may comprise the following steps:

2

3 a. exposing the test substance to a sample containing
4 PKB, to form a mixture;

5

6 b. exposing said mixture to a peptide comprising the
7 amino acid sequence defined above or a functional
8 equivalent thereof (usually in the presence of Mg^{2+}
9 and ATP); and

10

11 c. detecting whether (and, optionally, to what
12 extent) said peptide has been phosphorylated.

13

14 In this aspect the method of the invention can be used
15 to determine whether the substance being tested acts on
16 PKB or directly on GSK3. This can be done by comparing
17 the phosphorylation states of the peptide and PKB; if
18 the phosphorylation state of GSK3 is changed but that
19 of PKB is not then the substance being tested acts
20 directly on GSK3 without acting on PKB.

21 In a further aspect the present invention provides a
22 method of treatment of the human or non-human
23 (preferably mammalian) animal body, said method
24 comprising administering PKB, its analogues,
25 inhibitors, stimulators or functional equivalents
26 thereof to said body. Said method affects the
27 regulation of glycogen metabolism in the treated body.

28

29 The method of treatment of the present invention may be
30 of particular use in the treatment of type II diabetes
31 (where desirably an activator of PKB is used, so that
32 the down-regulation of GSK3 activity due to the action
33 of PKB is enhanced).

34

35 The method of treatment of the present invention may
36 alternatively be of particular use in the treatment of

1 cancer such as ovarian cancer (where desirably an
2 inhibitor of PKB is used, so that the down-regulation
3 of GSK3 activity due to the action of PKB is
4 depressed). Other cancers associated with
5 irregularities in the activity of PKB and/or GSK3 may
6 also be treated by the method, such as pancreatic
7 cancer, and breast cancer.

8
9 Stimulation of PKB with insulin increases activity
10 12-fold within 5 min and induces its phosphorylation at
11 Thr-308 and Ser-473. PKB transiently transfected into
12 cells can be activated 20-fold in response to insulin
13 and 46-fold in response to IGF-1 and also became
14 phosphorylated at Thr-308 and Ser-473. The activation
15 of PKB and its phosphorylation at both Thr-308 and
16 Ser-473 can be prevented by the phosphatidylinositol
17 (PI) 3-kinase inhibitor wortmannin. The
18 phosphorylation of threonine 308 and serine 473 act
19 synergistically to activate PKB.

20
21 MAPKAP kinase-2-phosphorylated PKB at Ser-473 in vitro
22 increases activity seven-fold, an effect that can be
23 mimicked (fivefold activation) by mutating Ser-473 to
24 Asp. Mutation of Thr-308 to Asp also increases PKB
25 activity five-fold and subsequent phosphorylation of
26 Ser-473 by MAPKAP kinase-2 stimulates activity a
27 further fivefold, an effect mimicked (18-fold
28 activation) by mutating both Thr-308 and Ser-473 to
29 Asp. The activity of the Asp-308/Asp-473 double mutant
30 was similar to that of the fully phosphorylated enzyme
31 and could not be activated further by insulin. Mutation
32 of Thr-308 to Ala did not prevent the phosphorylation
33 of transfected PKB at Ser-473 after stimulation of 293
34 cells with insulin or IGF-1, but abolished the
35 activation of PKB. Similarly, mutation of Ser-473 to
36 Ala did not prevent the phosphorylation of transfected

1 PKB at Thr-308 but greatly reduced the activation of
2 transfected PKB. This demonstrates that the activation
3 of PKB by insulin or IGF-1 results from the
4 phosphorylation of Thr-308 and Ser-473 and that
5 phosphorylation of both residues is preferred to
6 generate a high level of PKB activity in vitro or in
7 vivo. Also, phosphorylation of Thr-308 in vivo is not
8 dependent on the phosphorylation of Ser-473 or vice
9 versa, that the phosphorylation of Thr-308 and Ser-473
10 are both dependent on PI 3-kinase activity and suggest
11 that neither Thr-308 nor Ser-473 phosphorylation is
12 catalysed by PKB itself.

13

14 Thus, it is preferred that the present invention
15 incorporates the use of any agent which affects
16 phosphorylation of PKB at amino acids 308 and/or 473,
17 for example insulin, inhibitors of PI 3-kinase such as
18 wortmannin or the like. The use of PKB, itself altered
19 at amino acids 308 and/or 473 (eg by phosphorylation
20 and/or mutation) is also suitable.

21

22 In a variation of the method of the present invention,
23 stimulation or inhibition of PKB may be assessed by
24 monitoring the phosphorylation states of amino acids
25 308 and/or 473 on PKB itself.

26

27 Different isoforms of PKB may be used or targeted in
28 the present invention; for example PKB α , β or γ .

29

30 The present invention will now be described in more
31 detail in the accompanying examples which are provided
32 by way of non-limiting illustration, and with reference
33 to the accompanying drawings.

34

35 **Example 1: PKB influences GSK3 activity.**

36 Fig 1: a, L6 myotubes were incubated for 15 min with 2

1 mM 8-bromocyclic-AMP (8Br-cAMP) and then with 0.1 μ M
2 insulin (5 min). Both GSK3 isoforms were co-
3 immunoprecipitated from the lysates and assayed before
4 (black bars) and after (white bars) reactivation with
5 PP2A (Cross et al., 1994). The results are presented
6 relative to the activity in unstimulated cells, which
7 was 0.08 ± 0.006 U mg^{-1} ($n=10$).
8 *b, c*, The inhibition of GSK3 by insulin (0.1 μ M) is
9 unaffected by rapamycin (0.1 μ M) and PD 98059 (50 μ M),
10 but prevented by LY 294002 (100 μ M).
11
12 *b*, L6 myotubes were stimulated with insulin for the
13 times indicated with (filled triangle) or without
14 (filled circles) a 15 min preincubation with LY 294002,
15 and GSK3 measured as in *a*. The open circles show
16 experiments from insulin-stimulated cells where GSK3
17 was assayed after reactivation with PP2A (Cross et al.,
18 1994).
19
20 *c*, cells were incubated with rapamycin (triangles) or
21 rapamycin plus PD 98059 (circles) before stimulation
22 with insulin, and GSK3 activity measured as in *a*,
23 before (filled symbols) and after (open symbols)
24 pretreatment with PP2A.
25
26 *d, e*, L6 myotubes were incubated with 8Br-cAMP (15 min)
27 PD 98059 (60 min) or LY 294002 (15 min) and then with
28 insulin (5 min) as in *a-c*. Each enzyme was assayed
29 after immunoprecipitation from lysates, and the results
30 are presented relative to the activities obtained. In
31 the presence of insulin and absence of 8Br-cAMP, which
32 were 0.04 ± 0.005 U mg^{-1} (p42 MAP kinase, $n=6$) and $0.071 \pm$
33 0.004 U mg^{-1} (MAPKAP Kinase⁻¹, $n=6$).
34
35 All the results (\pm s.e.m.) are for at least three
36 experiments.

1 Monolayers of L6 cells were cultured, stimulated and
2 lysed as described previously (Cross et al., 1994).
3 p42 MAP kinase, MAPKAP kinase 1 or (GSK3- α plus GSK3- β)
4 were then immunoprecipitated from the lysates and
5 assayed with specific protein or peptide substrates as
6 described previously (Cross et al., 1994). One unit of
7 protein kinase activity was that amount which catalysed
8 the phosphorylation of 1 nmol of substrate in 1 min.
9 Where indicated, GSK3 in immunoprecipitates was
10 reactivated with PP2A (Cross et al., 1994).

11

12

13 Figure 2 Identification of PKB as the insulin-
14 stimulated, wortmannin-sensitive and PD
15 98059/rapamycin-insensitive Crosstide kinase in L6
16 myotubes.

17 a. Cells were incubated with 50 μ M PD 98059 (for 1
18 hour) and 0.1 μ M rapamycin (10 min), then stimulated
19 with 0.1 μ M insulin (5 min) and lysed (Cross et al.,
20 1994). The lysates (0.3 mg protein) were
21 chromatographed on Mono Q (5 x 0.16cm) and fractions
22 (0.05ml) were assayed for Crosstide kinase (filled
23 circles). In separate experiments insulin was omitted
24 (open circles) or wortmannin (0.1 μ M) added 10 min
25 before the insulin (filled triangles). The broken line
26 shows the NaCl gradient. Similar results were obtained
27 in six experiments.

28

29 b. Pooled fractions (10 μ l) 31-34 (lane 1), 35-38 (lane
30 2), 39-42 (lane 3), 43-45 (lane 4), 46-49 (lane 5) and
31 50-53 (lane 6) from a were electrophoresed on a 10%
32 SDS/polyacrylamide gel and immunoblotted with the C-
33 terminal anti-PKB α antibody. Marker proteins are
34 indicated. No immunoreactive species were present in
35 fractions 1-30 or 54-80.

36

1 c. L6 myotubes were stimulated with 0.1 μ M insulin and
2 PKB immunoprecipitated from the lysates (50 μ g protein)
3 essentially as described previously (Lazar et al.,
4 1995), using the anti-PH domain antibody and assayed
5 for Crosstide kinase (open circles). In control
6 experiments, myotubes were incubated with 0.1 μ M
7 rapamycin plus 50 μ M PD 98059 (open triangles) or 2 mM
8 8Br-cAMP (open squares), or 0.1 μ M wortmannin (filled
9 circles) or 100 μ M LY 294002 (filled triangles) before
10 stimulation with insulin.

11

12 d. As c, except that MAPKAP kinase-1 was
13 immunoprecipitated from the lysates and assayed with S6
14 peptide (filled circles). In control experiments,
15 cells were incubated with 0.1 μ M rapamycin plus 50 μ M
16 PD 98059 (filled triangles) or with 2 μ M 8BR-cAMP (open
17 circles) before stimulation with insulin. In c and d,
18 the error bars denote triplicate determinations, and
19 similar results were obtained in three separate
20 experiments.

21

22 Mono Q chromatography was performed as described
23 (Burgering et al., 1995), except that the buffer also
24 contained 1 mM EGTA, 0.1 mM sodium orthovanadate and
25 0.5% (w/v) Triton X-100. Two PKB α antibodies were
26 raised in rabbits against the C-terminal peptide
27 FPQFSYSASSTA and bacterially expressed PH domain of
28 PKB α . The C-terminal antibody was affinity purified
29 (Jones et al., 1991). The activity of PKB towards
30 Crosstide is threefold higher than its activity towards
31 histone H2B and 11-fold higher than its activity
32 towards myelin basic protein, the substrates used
33 previously to assay PKB. Other experimental details
34 and units of protein kinase activity are given in
35 Fig 1.

36

Figure 3 GSK3 is inactivated by PKB from insulin-stimulated L6 myotubes.

a. Cells were stimulated for 5 min with 0.1 μ M insulin, and PKB immunoprecipitated from 100 μ g of cell lysate and used to inactivate GSK3 isoforms essentially as described previously (Sutherland et al., 1993 and Sutherland et al., 1994). The black bars show GSK3 activity measured after incubation with MgATP and PKB as a percentage of the activity obtained in control incubations where PKB was omitted. In the absence of PKB, GSK3 activity was stable throughout the experiment. The white bars show the activity obtained after reactivation of GSK3 with PP2A (Embi et al., 1980). No inactivation of GSK3 occurred if insulin was omitted, or if wortmannin (0.1 μ M) was added 10 min before the insulin, or if the anti-PKB antibody was incubated with peptide immunogen (0.5 mM) before immunoprecipitation. The results (\pm s.e.m.) are for three experiments (each carried out in triplicate).

b. Inactivation of GSK3- β by HA-PKB α . Complementary DNA encoding HA-PKB α was transfected into COS-1 cells, and after stimulation for 15 min with 0.1 mM sodium pervanadate the tagged protein kinase was immunoprecipitated from 0.3 mg of lysate and incubated for 20 min with GSK3- β and MgATP. In control experiments, pervanadate was omitted, or wildtype (WT) PKB α replaced by vector (mock translation) or by a kinase-inactive mutant of PKB α in which Lys 179 was mutated to Ala (K179A). Similar results were obtained in three separate experiments. The levels of WT and K179A-PKB α in each immunoprecipitate were similar in each transfection.

In a GSK3- α and GSK3- β were partially purified, assayed, inactivated by PKB, and reactivated by PP2A

1 from rabbit skeletal muscle as described previously
2 (Sutherland et al., 1993 and Sutherland et al.,
3 1994). There was no reactivation in control
4 experiments in which okadaic acid (2 μ M) was added
5 before PP2A.

6
7 **Figure 4: Identification of the residues in GSK3**
8 **phosphorylated by PKB in vitro and in response to**
9 **insulin in L6 myotubes.**

10 a. GSK3- β was maximally inactivated by incubation with
11 PKB and Mg-[γ - 32 P]ATP and after SDS-PAGE, the 32 P-
12 labelled GSK3- β (M_r 47K) was digested with trypsin¹¹ and
13 chromatographed on a C₁₈ column (Sutherland et al.,
14 1993). Fractions (0.8 ml) were analysed for 32 P-
15 radioactivity (open circles), and the diagonal line
16 shows the acetonitrile gradient.

17
18 b. The major phosphopeptide from a (400 c.p.m.) was
19 subjected to solid-phase sequencing (Sutherland et al.,
20 1993), and 32 P-radioactivity released after each cycle
21 of Edman degradation is shown.

22
23 c. GSK3- α and GSK3- β were co-immunoprecipitated from
24 the lysates of 32 P-labelled cells, denatured in SDS,
25 subjected to SDS-PAGE, transferred to nitrocellulose
26 and autoradiographed (Saito et al., 1994). Lanes 1-3,
27 GSK3 isoforms immunoprecipitated from unstimulated
28 cells; lanes 4-6, GSK3 isoforms immunoprecipitated from
29 insulin-stimulated cells.

30
31 d. GSK3 isoforms from c. were digested with trypsin,
32 and the resulting phosphopeptides separated by
33 isoelectric focusing (Saito et al., 1994) and
34 identified by auto-radiography. Lanes 1 and 4 show the
35 major phosphopeptide resulting from in vitro
36 phosphorylation of GSK3- β by PKB and MAPKAP kinase-1,

1 respectively; lanes 2 and 5, the phosphopeptides
2 obtained from GSK3- β and GSK3- α , immunoprecipitated
3 from unstimulated cells; lanes 3 and 6, the
4 phosphopeptides obtained from GSK3- β and GSK3- α
5 immunoprecipitated from cells stimulated for 5 min with
6 0.1 μ M insulin; the arrow denotes the peptides whose
7 phosphorylation is increased by insulin. The pI values
8 of two markers, Patent Blue (2.4) and azurin (5.7) are
9 indicated.

10
11 In a. PKB α was immunoprecipitated with the C-terminal
12 antibody from the lysates (0.5 mg protein) of insulin-
13 stimulated L6 myotubes and used to phosphorylate GSK-
14 β^{12} . In c. three 10-cm diameter dishes of L6 myotubes
15 were incubated for 4 hours in HEPES-buffered saline
16 (Cross et al., 1994) containing 50 μ M PD 98059, 100 nM
17 rapamycin and 1.5 mCi ml⁻¹ ³²P-orthophosphate, stimulated
18 for 5 min with insulin (0.1 μ M) or buffer, and GSK3
19 isoforms co-immunoprecipitated from the lysates as in
20 Fig 1.

21 22 Discussion.

23 Inhibition of GSK3 induced by insulin in L6 myotubes
24 (Fig 1a-c) was unaffected by agents which prevented the
25 activation of MAPKAP kinase-1 (8-bromo-cyclic AMP, or
26 PD 98059 (Alessi et al., 1995), (Fig 1d,e) and/or p70^{S6k}
27 (rapamycin (Kuo et al., 1992)) (Cross et al., 1994),
28 suggesting that neither MAPKAP kinase-1 nor p70^{S6k} are
29 essential for this process. However, the
30 phosphorylation and inhibition of GSK3- β after phorbol
31 ester treatment (Stambolic et al., 1994) is enhanced by
32 coexpression with MAPKAP kinase 1 in HeLa S3 cells,
33 whereas in NIH 3T3 cells the EGF-induced inhibition of
34 GSK3- α and GSK3- β (Saito et al., 1994) is largely
35 suppressed by expression of a dominant-negative mutant
36 of MAP kinase kinase-1 (Elgar et al., 1995). MAPKAP

1 kinase-1 may therefore mediate the inhibition of GSK3
2 by agonists which are much more potent activators of
3 the classical MAP kinase pathway than is insulin.

4
5 To identify the insulin-stimulated protein kinase that
6 inhibits GSK3 in the presence of rapamycin and PD
7 98059, L6 myotubes were incubated with both compounds
8 and stimulated with insulin. The lysates were then
9 chromatographed on Mono Q and the fractions assayed
10 with "Crosstide" (GRPRTSSFAEG), a peptide corresponding
11 to the sequence in GSK3 surrounding the serine
12 (underlined) phosphorylated by MAPKAP kinase-1 and p70^{S6k}
13 (Ser 21 in GSK3- α (Sutherland et al., 1994) and Ser 9
14 in GSK3- β (Sutherland et al 1993)). Three peaks of
15 Crosstide kinase activity were detected, which were
16 absent if insulin stimulation was omitted or if the
17 cells were first preincubated with the PI 3-kinase
18 inhibitor wortmannin (Fig 2a). Wortmannin (Cross et
19 al., 1994 and Welsh et al 1994), and the structurally
20 unrelated PI 3-kinase inhibitor LY 294002 (ref 19);
21 (Fig 1b), both prevent the inhibition of GSK3 by
22 insulin.

23
24 The protein kinases PKB- α , PKB- β and PKB γ are Ser/Thr-
25 specific and cellular homologues of the viral oncogene
26 v-akt (Coffer et al., 1991, Jones et al 1991, Ahmed et
27 al 1995 and Cheng et al., 1992). These enzymes have
28 recently been shown to be activated in NIH 3T3, Rat-1
29 or Swiss 3T3 cells in response to growth factors or
30 insulin, activation being suppressed by blocking the
31 activation of PI 3-kinase in different ways (Franke et
32 al., 1995 and Burgering et al., 1995). All three peaks
33 of Crosstide kinase (Fig 2a), but no other fraction
34 from Mono Q, showed the characteristic multiple bands
35 of PKB (relative molecular mass, M_r 58K, 59K or 60K)
36 that have been observed in other cells, when

1 immunoblotting was performed with an antibody raised
2 against the carboxyl-terminal peptide of PKB- α (Fig
3 2b). The more slowly migrating forms represent more
4 highly phosphorylated protein, and are converted to the
5 fastest migrating species by treatment with
6 phosphatases. Phosphatase treatment also results in
7 the inactivation of PKB (Burgering et al., 1995) and
8 the complete loss of Crosstide kinase activity (data
9 not shown). Of the Crosstide kinase activity in peaks
10 2 and 3 from Mono Q, 70-80% was immunoprecipitated by a
11 separate antibody raised against the amino-terminal
12 pleckstrin homology (PH) domain of PKB- α . The C-
13 terminal antibody also immunoprecipitated PKB activity
14 specifically from peaks 2 and 3, but was less effective
15 than the anti-PH-domain antibody. Peak-1 was hardly
16 immunoprecipitated by either antibody and may represent
17 PKB- β . An immunoprecipitating anti-MAPKAP kinase-1
18 antibody (Cross et al., 1994) failed to deplete any of
19 the Crosstide kinase activity associated with peaks 1,
20 2 or 3.

21
22 Insulin stimulation of L6 myotubes increased PKB
23 activity by more than tenfold (Fig 2c), and activation
24 was blocked by wortmannin or LY 294002, but was
25 essentially unaffected by 8-bromo-cyclic AMP or
26 rapamycin plus PD 98059 (Fig 2c). The half-time ($t_{0.5}$)
27 or activation of PKB (1 min) was slightly faster than
28 that for inhibition of GSK3 (2 min) (Cross et al.,
29 1994). In contrast, the activation of MAPKAP kinase-1
30 (Fig 2d) and p70^{S6k} (not shown) was slower ($t_{0.5} > 5$ min).
31 Activation of MAPKAP kinase-1 was prevented by 8-bromo-
32 cyclic AMP or PD 98059 (Fig 2d), and activation of p70^{S6k}
33 by rapamycin (Cross et al., 1994). Akt/RAC
34 phosphorylated the Ser in the Crosstide equivalent to
35 Ser 21 in GSK3- α and Ser 9 in GSK3- β (data not shown).
36

1 PKB from insulin-stimulated L6 myotubes (but not from
2 unstimulated or wortmannin-treated cells) inactivated
3 GSK3- α and GSK3- β *in vitro*, and inhibition was reversed
4 by the Ser/Thr-specific protein phosphatase PP2A (Embi
5 et al., 1980) (Fig 3a). To further establish that
6 inactivation was catalysed by PKB, and not by a co-
7 immunoprecipitating protein kinase, haemagglutinin-
8 tagged PKB- α (HA-PKB) was transfected into COS-1 cells
9 and activated by stimulation with pervanadate, which is
10 the strongest inducer of PKB activation in this system.
11 The HA-PKB inactivated GSK3- β , but not if treatment
12 with pervanadate was omitted or if wild-type HA-PKB was
13 replaced with a "kinase inactive" mutant (Fig 3b).

14
15 The inactivation of GSK3- β by PKB *in vitro* was
16 accompanied by the phosphorylation of one major tryptic
17 peptide (Fig 4a) which coeluted during C_{18}
18 chromatography (Sutherland et al., 1993) and
19 isoelectric focusing with that obtained after
20 phosphorylation by MAPKAP kinase-1 (Fig 4d).
21 Stimulation of L6 myotubes with insulin (in the
22 presence of rapamycin and PD 98059) increased the ^{32}P -
23 labelling of GSK3- α and GSK3- β by 60-100% (Fig 4c) and
24 increased the ^{32}P -labelling of the same tryptic peptides
25 labelled *in vitro* (Fig 4d). Sequence analyses
26 established that the third residue of these,
27 corresponding to Ser 9 (GSK3- β) or Ser 21 (GSK3- α), was
28 the site of phosphorylation in each phosphopeptide,
29 both *in vitro* (Fig 4b) and *in vivo* (not shown). The
30 ^{32}P -labelling of other (more acidic) tryptic
31 phosphopeptides was not increased by insulin (Fig 4d).
32 These peptides have been noted previously in GSK3 from
33 A431 cells and shown to contain phosphoserine and
34 phosphotyrosine (Saito et al., 1994).

35
36 PKC- δ , ϵ and ζ are reported to be activated by

1 mitogens, and PKC- ζ activity is stimulated *in vitro* by
2 several inositol phospholipids, including PI(3,4,5)P₃,
3 the product of the PI 3-kinase reaction (Andjelkovic et
4 al., 1995). However, purified PKC- ϵ (Palmer et al.,
5 1995), PKC- δ and PKC- ζ (data not shown) all failed to
6 inhibit GSK3- α or GSK3- β *in vitro*. Moreover, although
7 PKC- α , β 1 and γ inhibit GSK3- β *in vitro* (Palmer et al.,
8 1995), GSK3- α is unaffected, while their downregulation
9 in L6 myotubes by prolonged incubation with phorbol
10 esters abolishes the activation of MAPKAP kinase-1 in
11 response to subsequent challenge with phorbol esters,
12 but has no effect on the inhibition of GSK3 by insulin
13 (not shown).

14
15 Taken together, our results identify GSK3 as a
16 substrate for PKB. The stimulation of glycogen
17 synthesis by insulin in skeletal muscle involves the
18 dephosphorylation of Ser residues in glycogen synthase
19 that are phosphorylated by GSK3 *in vitro* (Parker et
20 al., 1983). Hence the 40-50% inhibition of GSK3 by
21 insulin, coupled with a similar activation of the
22 relevant glycogen synthase phosphatase (Goode et al.,
23 1992), can account for the stimulation of glycogen
24 synthase by insulin in skeletal muscle (Parker et al.,
25 1983) or L6 myotubes (Goode et al., 1992). The
26 activation of glycogen synthase and the resulting
27 stimulation of glycogen synthesis by insulin in L6
28 myotubes is blocked by wortmannin, but not by PD 98059
29 (Dent et al., 1990), just like the activation of
30 Akt/RAC and inhibition of GSK3. However, GSK3 is
31 unlikely to be the only substrate of PKB *in vivo*, and
32 identifying other physiologically relevant substrates
33 will be important because PKB β is amplified and over-
34 expressed in many ovarian neoplasms (Cheng et al.,
35 1992).

36

1 **Example 2: Activation of PKB by insulin in L6 myotubes**
2 **is accompanied by phosphorylation of residues Thr-308**
3 **and Ser-473.** Insulin induces the activation and
4 phosphorylation of PKB α in L6 myotubes. Three 10 cm
5 dishes of L6 myotubes were ^{32}P -labelled and treated for
6 10 min with or without 100 nM wortmannin and then for 5
7 min with or without 100 nM insulin. PKB α was
8 immunoprecipitated from the lysates and an aliquot
9 (15%) assayed for PKB α activity (Fig 5A). The
10 activities are plotted \pm SEM for 3 experiments relative
11 to PKB α derived from unstimulated cells which was 10
12 mU/mg. The remaining 85% of the immunoprecipitated PKB α
13 was alkylated with 4-vinylpyridine, electrophoresed on
14 a 10% polyacrylamide gel (prepared without SDS to
15 enhance the phosphorylation-induced decrease in
16 mobility) and autoradiographed. The positions of the
17 molecular mass markers glycogen phosphorylase (97 kDa),
18 bovine serum albumin (66 kDa) and ovalbumin (43 kDa)
19 are marked.

20
21 Under these conditions, insulin stimulation resulted in
22 a 12-fold activation of PKB α (Fig 5A) and was
23 accompanied by a 1.9 ± 0.3 -fold increase in
24 ^{32}P -labelling (4 experiments) and retardation of its
25 mobility on SDS-polyacrylamide gels (Fig 5B). The
26 activation of PKB α , the increase in its ^{32}P -labelling
27 and reduction in electrophoretic migration were all
28 abolished by prior incubation of the cells with 100 nM
29 wortmannin. Phosphoamino acid analysis of the whole
30 protein revealed that ^{32}P -labelled PKB α was
31 phosphorylated at both serine and threonine residues
32 and that stimulation with insulin increased both the
33 ^{32}P -labelling of both phosphoamino acids (data not
34 shown).

35
36 **Fig. 6. Insulin stimulation of L6 myotubes induces the**

1 phosphorylation of two peptides in PKB α . Bands
2 corresponding to ^{32}P -labelled PKB α from Fig 5B were
3 excised from the gel, treated with 4-vinylpyridine to
4 alkylate cysteine residues, digested with trypsin and
5 chromatographed on a Vydac 218TP54 C18 column
6 (Separations Group, Hesperia, CA) equilibrated with
7 0.1% (by vol) trifluoroacetic acid (TFA), and the
8 columns developed with a linear acetonitrile gradient
9 (diagonal line). The flow rate was 0.8 ml / min and
10 fractions of 0.4 ml were collected (A) tryptic peptide
11 map of ^{32}P -labelled PKB α from unstimulated L6 myotubes;
12 (B) tryptic peptide map of ^{32}P -labelled PKB α from
13 insulin-stimulated L6 myotubes; (C) tryptic peptide map
14 of ^{32}P -labelled PKB α from L6 myotubes treated with
15 wortmannin prior to insulin. The two major ^{32}P -labelled
16 peptides eluting at 23.7% and 28% acetonitrile are
17 named Peptide A and Peptide B, respectively. Similar
18 results were obtained in four (A and B) and two (C)
19 experiments.

20
21 No major ^{32}P -labelled peptides were recovered from
22 ^{32}P -labelled PKB α derived from unstimulated L6 myotubes
23 (Fig 6A) indicating that, in the absence of insulin,
24 there was a low level phosphorylation at a number of
25 sites. However, following stimulation with insulin, two
26 major ^{32}P -labelled peptides were observed, termed A and
27 B (Fig 6B), whose ^{32}P -labelling was prevented if the
28 myotubes were first preincubated with wortmannin (Fig
29 6C).

30
31 Fig 7. Identification of the phosphorylation sites in
32 peptides A and B. (A) Peptides A and B from Fig5B
33 (1000cpm) were incubated for 90min at 110°C in 6M HCl,
34 electrophoresed on thin layer cellulose at pH 3.5 to
35 resolve orthophosphate (Pi), phosphoserine (pS),
36 phosphthreonine (pT) and phosphotyrosine (pY) and

1 autoradiographed. (B) Peptide A (Fig 5B) obtained from
2 50 10 cm dishes of ^{32}P -labelled L6 myotubes was further
3 purified by chromatography on a microbore C18 column
4 equilibrated in 10 mM ammonium acetate pH 6.5 instead
5 of 0.1% TFA. A single peak of ^{32}P -radioactivity was
6 observed at 21% acetonitrile which coincided with a
7 peak of 214 nm absorbance. 80% of the sample (1 pmol)
8 was analysed on an Applied Biosystems 476A sequencer to
9 determine the amino acid sequence, and the
10 phenylthiohydantoin (Pth) amino acids identified after
11 each cycle of Edman degradation are shown using the
12 single letter code for amino acids. The residues in
13 parentheses were not present in sufficient amounts to
14 be identified unambiguously. To identify the site(s)
15 of phosphorylation, the remaining 20% of the sample
16 (600 cpm) was then coupled covalently to a Sequelon
17 arylamine membrane and analysed on an Applied
18 Biosystems 470A sequencer using the modified programme
19 described by Stokoe et al. (1992). ^{32}P radioactivity was
20 measured after each cycle of Edman degradation. (C)
21 Peptide B from Fig 2B (800 cpm) was subjected to solid
22 phase sequencing as in (B).

23
24 Peptide A was phosphorylated predominantly on serine
25 while peptide B was labelled on threonine (Fig 7A).
26 Amino acid sequencing established that peptide A
27 commenced at residue 465. Only a single burst of
28 ^{32}P -radioactivity was observed after the eighth cycle of
29 Edman degradation (Fig 7B), demonstrating that insulin
30 stimulation of L6 myotubes had triggered the
31 phosphorylation of PKB α at Ser-473, which is located 9
32 residues from the C-terminus of the protein.
33 Phosphopeptide B was only recovered in significant
34 amounts if ^{32}P -labelled PKB α was treated with
35 4-vinylpyridine prior to digestion with trypsin,
36 indicating that this peptide contained a cysteine

1 residue(s), and a single burst of 32p radioactivity was
2 observed after the first cycle of Edman degradation
3 (Fig 7C). This suggested that the site of
4 phosphorylation was residue 308, since it is the only
5 threonine in PKBa that follows a lysine or arginine
6 residue and which is located in a tryptic peptide
7 containing a cysteine residue (at position 310). The
8 acetonitrile concentration at which phosphopeptide B is
9 eluted from the C18 column (28%) and its isoelectric
10 point (4.0) are also consistent with its assignment as
11 the peptide comprising residues 308-325 of PKBa. The
12 poor recoveries of Peptide B during further
13 purification at pH 6.5 prevented the determination of
14 its amino acid sequence, but further experiments
15 described below using transiently transfected 293 cells
16 established that this peptide does correspond to
17 residues 308-325 of PKBa.

18
19 **Fig 8: Mapping the phosphorylation sites of PKBa in**
20 **transiently transfected 293 cells.** 293 cells were
21 transiently transfected with DNA constructs expressing
22 wild type PKBa, or a haemagglutinin epitope-tagged PKBa
23 encoding the human protein, such as HA-KD PKBa, HA-473A
24 PKBa or HA-308A PKBa. After treatment for 10 min with
25 or without 100 nM wortmannin, the cells were stimulated
26 for 10 min with or without 100 nM insulin or 50 ng/ml
27 IGF- 1 in the continued presence of wortmannin. PKBa
28 was immunoprecipitated from the lysates and assayed,
29 and activities corrected for the relative levels of
30 expression of each HA-PKBa. The results are expressed
31 relative to the specific activity of wild type HA-PKBa
32 from unstimulated 293 cells (2.5 ± 0.5 U/mg). (B) 20 μ g
33 of protein from each lysate was electrophoresed on a 10
34 % SDS/polyacrylamide gel and immunoblotted using
35 monoclonal HA-antibody. The molecular markers are those
36 used in Fig 5B.

1 Fig 9: IGF-1 stimulation of 293 cells induces the
2 phosphorylation of two peptides in transfected HA-PKB α .
3 293 cells transiently transfected with wild type HAPKB α
4 DNA constructs were 32 P-labelled, treated for 10 min
5 without (A,B) or with (C) 100 nM wortmannin and then
6 for 10 min without (A) or with (B, C) 50 ng/ml IGF-1.
7 The 32 p labelled HA-PKB α was immunoprecipitated from
8 the lysates, treated with 4-vinylpyridine,
9 electrophoresed on a 10% polyacrylamide gel, excised
10 from the gel and digested with trypsin. Subsequent
11 chromatography on a C₁₈ column resolved four major
12 phosphopeptides termed C, D, E and F. Similar results
13 were obtained in 6 separate experiments for (A) and
14 (B), and in two experiments for (C).

15
16 Stimulation with insulin and IGF-1 resulted in 20-fold
17 and 46-fold activation of transfected PKB α ,
18 respectively (Fig 8A), the half time for activation
19 being 1 min, as found with other cells. Activation of
20 PKB α by insulin or IGF-1 was prevented by prior
21 incubation with wortmannin (Fig 8A) and no activation
22 occurred if 293 cells were transfected with vector
23 alone and then stimulated with insulin or IGF-1 (data
24 not shown).

25
26 Two prominent 32 P-labelled peptides were present in
27 unstimulated 293 cells (Fig 9A). One, termed Peptide C,
28 usually eluted as a doublet at 20-21% acetonitrile
29 while the other, termed Peptide F, eluted at 29.7%
30 acetonitrile. Stimulation with insulin or IGF-1 did
31 not affect the 32 P-labelling of Peptides C and F (Figs
32 9A & B), but induced the 32 P-labelling of two new
33 peptides, termed D (23.4% acetonitrile) and E
34 (28% acetonitrile), which eluted at the same
35 acetonitrile concentrations as peptides A and B from L6
36 myotubes (Fig 6B) and had the same isoelectric points

1 (7.2 and 4.0, respectively). Treatment of 293 cells
2 expressing HA-PKB α with 100 nM wortmannin, prior to
3 stimulation with IGF-1, prevented the phosphorylation
4 of Peptides D and E, but had no effect on the 32p
5 labelling of Peptides C and F (Fig 9C).

6
7 Peptides C, D, E and F were further purified by re-
8 chromatography on the C18 column at pH 6.5 and
9 sequenced. Peptides C gave rise to three separate (but
10 closely eluting) ³²P-labelled peptides (data not shown).
11 Amino acid sequencing revealed that all three commenced
12 at residue 122 of PKB α and that Ser-124 was the site of
13 phosphorylation (Fig 10A). Peptide D only contained
14 phosphoserine and, as expected, corresponded to the
15 PKB α tryptic peptide commencing at residue 465 that was
16 phosphorylated at Ser-473 (Fig 10B). Peptide E, only
17 contained phosphothreonine and amino acid sequencing
18 demonstrated that it corresponded to residues 308-325,
19 the phosphorylation site being Thr-308 (Fig 10C).
20 Peptide F only contained phosphothreonine and
21 corresponded to the peptide commencing at residue 437
22 of PKB α phosphorylated at Thr-450 (Fig 10D).

23
24 In the presence of phosphatidylserine, PKB α binds to
25 PIP3 with submicromolar affinity (James et al., 1996,
26 Frech et al., 1996). Phosphatidyl 4,5-bisphosphate and
27 phosphatidyl 3,4 bisphosphate bind to PKB α with lower
28 affinities and PI 3,5 bisphosphate and PI 3 phosphate
29 did not bind at all under these conditions (James et
30 al., 1996). The region of PKB α that interacts with PIP3
31 is almost certainly the PH domain, because the isolated
32 PH domain binds PIP3 with similar affinity to PKB α
33 itself (Frech et al., 1996) and because the PH domain
34 of several other proteins, such as the PH-domains of,
35 β -spectrin and phospholipase C1, are known to interact
36 specifically with other phosphoinositides (Hyvonen et

1 al., 1995 and Lemmon et al., 1995).

2

3 The experiments described above were repeated using
4 insulin instead of IGF-1. The results were identical,
5 except that the ^{32}P -labelling of Peptides D and E was
6 about 50% of the levels observed with IGF-1 (data not
7 shown). This is consistent with the two-fold lower
8 level of activation of PKB α by insulin compared with
9 IGF-1 (Fig 7A).

10

11 Example 3: MAPKAP kinase-2 phosphorylates Ser-473 of
12 PKB α causing partial activation. Ser-473 of PKB α lies
13 in a consensus sequence Phe-x-x-Phe/Tyr-Ser/Thr-Phe/Tyr
14 found to be conserved in a number of protein kinases
15 that participate in signal transduction pathways
16 (Pearson et al. 1995). In order to identify the Ser-473
17 kinase(s) we therefore chromatographed rabbit skeletal
18 muscle extracts on CM-Sephadex, and assayed the
19 fractions for protein kinases capable of
20 phosphorylating a synthetic peptide corresponding to
21 residues 465 to 478 of PKB α . These studies identified
22 an enzyme eluting at 0.3 M NaCl which phosphorylated
23 the peptide 465-478 at the residue equivalent to
24 Ser-473 of PKB α . The Ser473 kinase co-eluted from
25 CM-Sephadex with MAP kinase-activated protein (MAPKAP)
26 kinase-2, (Stokoe et al, 1992) which is a component of
27 a stress and cytokine-activated MAP kinase cascade
28 (Rouse et al, 1994; Cuenda et al, 1995). The Ser-473
29 kinase continued to cofractionate with MAPKAPkinase-2
30 through phenyl-Sepharose, heparin-Sepharose, Mono S and
31 Mono Q and was immunoprecipitated quantitatively by an
32 anti-MAPKAP kinase-2 antibody (Gould et al, 1995)
33 demonstrating that MAPKAP kinase-2 was indeed the
34 Ser-473 kinase we had purified.

35

36 Figure 11. HA-PKB α was immunoprecipitated from the

1 lysates of unstimulated COS-1 cells expressing these
2 constructs. (A) 0.5 μ g of immunoprecipitated HA-PKB α
3 was incubated with MAPKAP kinase-2 (50 U/ml), 10 mM
4 magnesium acetate and 100 mM [γ ³²P]ATP in a total of 40
5 μ l of Buffer B. At various times, aliquots were removed
6 and either assayed for PKB α activity (open circles) or
7 for incorporation of phosphate into PKB α (closed
8 circles). Before measuring PKB α activity, EDTA was
9 added to a final concentration of 20 mM to stop the
10 reaction, and the immunoprecipitates washed twice with
11 1.0 ml of buffer B containing 0.5 M NaCl, then twice
12 with 1.0 ml of Buffer B to remove MAPKAP kinase-2. The
13 results are presented as \pm SEM for six determinations
14 (two separate experiments) and PKB α activities are
15 presented relative to control experiments in which
16 HA-PKB α was incubated with MgATP in the absence of
17 MAPKAP kinase-2 (which caused no activation).
18 Phosphorylation was assessed by counting the
19 ³²P-radioactivity associated with the band of PKB α after
20 SDS/polyacrylamide gel electrophoresis. The open
21 triangles show the activity of immunoprecipitated HA-KD
22 PKB α phosphorylated by MAPKAP kinase-2. (B) HA-PKB α
23 phosphorylated for 1 h with MAPKAP kinase-2 and ³²P-
24 γ -ATP as in (A) was digested with trypsin and
25 chromatographed on a C18 column as described in the
26 legend for Fig 2. (C) The major ³²P-labelled peptide
27 from (B) was analysed on the 470A sequencer as in Fig 3
28 to identify the site of phosphorylation.
29
30 Bacterially expressed MAPKAP kinase-2 phosphorylated
31 wild type HA-PKB α or the catalytically inactive mutant
32 HA-PKB α in which Lys- 179 had been mutated to Ala (data
33 not shown) to a level approaching 1 mol per mole
34 protein (Fig 11A). Phosphorylation of wild-type PKB α
35 was paralleled by a seven-fold increase in activity,
36 whereas phosphorylation of the catalytically inactive

1 mutant did not cause any activation (Fig 11A). No
2 phosphorylation or activation of wild type HA-PKB α
3 occurred if MAPKAP kinase-2 or MgATP was omitted from
4 the reaction (data not shown). Wild type HA-PKB α that
5 had been maximally activated with MAPKAP kinase-2, was
6 completely dephosphorylated and inactivated by
7 treatment with protein phosphatase 2A (data not shown).

8
9 HA-PKB α that had been maximally phosphorylated with
10 MAPKAP kinase-2 was digested with trypsin and C18
11 chromatography revealed a single major ^{32}P -labelled
12 phosphoserine-containing peptide (Fig 11B). This
13 peptide eluted at the same acetonitrile concentration
14 (Fig 11B) and had the same isoelectric point of 7.2
15 (data not shown) as the ^{32}P labelled tryptic peptide
16 containing Ser-473 (compare Figs 11B and 6B). Solid
17 phase sequencing gave a burst of ^{32}P -radioactivity after
18 the eighth cycle of Edman degradation (Fig 11C),
19 establishing that Ser-473 was the site of
20 phosphorylation. The same ^{32}P -peptide was obtained
21 following tryptic digestion of catalytically inactive
22 HA-KD PKB α that had been phosphorylated with MAPKAP
23 kinase-2 (data not shown).

24
25 Example 4: Phosphorylation of Thr-308 and Ser-473
26 causes synergistic activation of PKB α . The experiments
27 described above demonstrated that phosphorylation of
28 Ser-473 activates PKB α in vitro but did not address the
29 role of phosphorylation of Thr-308, or how
30 phosphorylation of Thr-308 might influence the effect
31 of Ser-473 phosphorylation on activity, or vice versa.
32 We therefore prepared haemagglutinin (HA)-tagged PKB α
33 DNA constructs in which either Ser-473 or Thr-308 would
34 be changed either to Ala (to block the effect of
35 phosphorylation) or to Asp (to try and mimic the effect
36 of phosphorylation).

Fig 12. Activation of HA-PKB α mutants in vitro by MAPKAP kinase-2. (A) Wild type and mutant HA-PKB α proteins were immunoprecipitated from the lysates of unstimulated COS-1 cells expressing these constructs and incubated for 60 min with MgATP in the absence (-, filled bars) or presence (+, hatched bars) of MAPKAP kinase-2 and MgATP (50 U/ml). The PKB α protein was expressed as similar levels in each construct and specific activities are presented relative to wild type HA-PKB α incubated in the absence of MAPKAP kinase-2 (0.03 U/mg). The results are shown as the average \pm SEM for 3 experiments. (B) 20 μ g of protein from each lysate was electrophoresed on a 10 % SDS/polyacrylamide gel and immunoblotted using monoclonal HA-antibody.

All the mutants were expressed at a similar level in serum-starved COS-1 cells (data not shown) and the effects of maximally phosphorylating each of them at Ser-473 is shown in Fig 12A. Before phosphorylation with MAPKAP kinase-2 the activity of HA-473A PKB α was similar to that of unstimulated wild type HA-PKB α and, as expected, incubation with MAPKAP kinase-2 and MgATP did not result in any further activation of HA-473A PKB α . In contrast, the activity of HA-473D PKB α was five-fold to six-fold higher than that of unstimulated wild type HAPKB α protein, and similar to that of wild-type HA-PKB α phosphorylated at Ser-473. As expected, HA-473D PKB α was also not activated further by incubation with MAPKAP kinase-2 and MgATP. The activity of HA-308A PKB α was about 40% that of the unstimulated wild type enzyme, and after phosphorylation with MAPKAP kinase-2 is activity increased to a level similar to that of wild type HA-PKB α phosphorylated at Ser-473. Interestingly, HA-308D PKB α which (like HA-473D PK) was five-fold more active than dephosphorylated wild type HA-PKB α , was

1 activated dramatically by phosphorylation of Ser-473.
2 After incubation with MAPKAP kinase-2 and MgATP, the
3 activity of HA-308D PKB α was nearly five-fold higher
4 than that of wild type HA-PKB α phosphorylated at
5 Ser-473 (Fig 12B). These results suggested that the
6 phosphorylation of either Thr-308 or Ser-473 leads to
7 partial activation of PKB α in vitro, and that
8 phosphorylation of both residues results in a
9 synergistic activation of the enzyme. This idea was
10 supported by further experiments in which both Thr-308
11 and Ser-473 were changed to Asp. When this double
12 mutant was expressed in COS-1 cells it was found to
13 possess an 18-fold higher specific activity than the
14 dephosphorylated wild type protein. As expected, the
15 activity of this mutant was not increased further by
16 incubation with MAPKAP kinase-2 and MgATP (Fig 12B).

17
18 Example 5: Phosphorylation of both Thr-308 and Ser-473
19 is required for a high level of activation of PKB α in
20 vivo.

21
22 Fig 9. Effect of mutation of PKB α on its activation by
23 insulin in 293 cells. (A) 293 cells were transiently
24 transfected with DNA constructs expressing wild type
25 PKB α , HA-D473- PKB α , and HA-308D/473D-PKB α . After
26 treatment for 10 min with or without 100 nM wortmannin,
27 cells were stimulated for 10 min with or without 100 nM
28 insulin in the continued presence of wortmannin. PKB α
29 was immunoprecipitated from the lysates and assayed,
30 and activities corrected for the relative levels of
31 HA-PKB α expression as described in the methods. The
32 results are expressed relative to the specific activity
33 of wild type HA-PKB α obtained from unstimulated 293
34 cells. (B) 20 μ g of protein from each lysate was
35 electrophoresed on a 10 % SDS/polyacrylamide gel and
36 immunoblotted using monoclonal HA-antibody.

1 The basal level of activity of HA-473A PKB α derived
2 from unstimulated cells was similar to that of wild
3 type PKB α (Fig 8A). Stimulation of 293 cells expressing
4 HA-473A PKB α with insulin or IGF-1 increased the
5 activity of this mutant three-fold and five-fold
6 respectively; i.e. to 15% of the activity of wild type
7 HA-PKB α which had been transiently expressed and
8 stimulated under identical conditions. The basal
9 activity of HA-308A PKB α in unstimulated cells was also
10 similar to that of wild type HA-PKB α derived from
11 unstimulated cells, but virtually no activation of this
12 mutant occurred following stimulation of the cells with
13 insulin or IGF-1. These data are consistent with in
14 vitro experiments and indicate that maximal activation
15 of PKB α requires phosphorylation of both Ser-473 and
16 Thr-308 and that phosphorylation of both residues
17 results in a synergistic activation of the enzyme.
18 Consistent with these results, HA-473D PKB α displayed
19 five-fold higher activity and the HA-308D/HA473D double
20 mutant 40-fold higher activity than wild type HA-PKB α
21 when expressed in unstimulated cells. Following
22 stimulation with insulin, HA-473D PKB α was activated to
23 a level similar to that observed with the wildtype
24 enzyme, while the HA-308D/HA-473D double mutant could
25 not be activated further (Fig 13). As expected,
26 activation of HA-473D PKB α by insulin was prevented by
27 wortmannin, and the activity of the HA-308D/ HA-473D
28 double mutant was resistant to wortmannin (Fig 13).

29
30 Example 6: Phosphorylation of Thr-308 is not dependent
31 on phosphorylation of Ser-473 or vice versa (in 293
32 cells). (Fig 10) A 10 cm dish of 293 cells were
33 transfected with either HA-308A PKB α or HA-473A PKB α ,
34 ³²P-labelled, then stimulated for 10 min with either
35 IGF-1 (50 ng/ml) or buffer. The ³²P-labelled PKB α
36 mutants were immunoprecipitated from the lysates,

1 treated with 4-vinylpyridine, electrophoresed on a 10%
2 polyacrylamide gel, excised from the gel and digested
3 with trypsin, then chromatographed on a C18 column.
4 The tryptic peptides containing the phosphorylated
5 residues Ser-124, Thr-308, Thr-450, Ser-473 are marked
6 and their assignments were confirmed by phosphoamino
7 acid analysis and sequencing to identify the sites of
8 phosphorylation (data not shown). The phosphopeptides
9 containing Thr-308 and Ser-473 were absent if
10 stimulation with IGF-1 was omitted, while the
11 phosphopeptides containing Ser-124 and Thr-450 were
12 present at similar levels as observed with wild-type
13 PKB α (see Fig 9A). Similar results were obtained in 3
14 separate experiments.

15
16 These experiments demonstrated that IGF-1 stimulation
17 induced the phosphorylation of HA-473A PKB α at Thr-308,
18 and the phosphorylation of HA-308A PKB α at Ser-473.
19 Similar results were obtained after stimulation with
20 insulin rather than IGF-I.

21
22 Example 7: IGF-1 or insulin induces phosphorylation of
23 Thr-308 and Ser-473 in a catalytically inactive mutant
24 of PKB α .

25
26 Fig 15. The catalytically inactive PKB α mutant
27 (HA-KD-PKB α) expressed in 293 cells is phosphorylated
28 at Thr-308 and Ser-473 after stimulation with IGF-1.
29 Each 10 cm dish of 293 cells transiently transfected
30 with HA-KD-PKB α DNA constructs was 32 P-labelled and
31 incubated for 10 min with buffer (A), 50 ng/ml IGF-1
32 (B) or 100 nM insulin (C). The 32 P-labelled HA-KD-PKB α
33 was immunoprecipitated from the lysates, treated with 4
34 vinylpyridine, electrophoresed on a 10% polyacrylamide
35 gel, excised from the gel and digested with trypsin,
36 then chromatographed on a C18 column. The tryptic

1 peptides containing the phosphorylated residues
2 Ser-124, Thr-308, Thr-450 and Ser-473 are marked.
3 Similar results were obtained in 3 separate experiments
4 for (A) and (B), and in two experiments for (C).
5

6 This kinase dead" mutant of PKB α , termed HA-KD-PKB α , in
7 which Lys-179 was changed to Ala (see above) was
8 transiently expressed in 293 cells and its level of
9 expression found to be several-fold lower than that of
10 wild type HA-PKB α expressed under identical conditions
11 (Fig 8B). As expected, no PKB α activity was detected
12 when 293 cells expressing HA-KD-PKB α , were stimulated
13 with insulin or IGF-1 (Fig 7A).
14

15 293 cells that had been transiently transfected with
16 HA-KD-PKB α were 32 P-labelled, then stimulated with
17 buffer, insulin or IGF-1. and sites on PKB α
18 phosphorylated under these conditions were mapped. In
19 contrast to wild type HA-PKB α from unstimulated 293
20 cells (Fig 9), HA-KD PKB α was phosphorylated to a much
21 lower level at Ser-124, but phosphorylated similarly at
22 Thr-450 (Fig 15A). Following stimulation with IGF-1
23 (Fig 15B) or insulin (Fig 14C) HA-KD-PKB α became
24 phosphorylated at the peptides containing Thr-308 and
25 Ser-473, the extent of phosphorylation of these sites
26 being at least as high as wild type PKB α . Amino acid
27 sequencing of these peptides established that they were
28 phosphorylated at Thr-308 and Ser-473, respectively.
29

30 The above examples establish that PKB influences GSK3
31 activity; that Thr-308 and Ser-473 are the major
32 residues in PKB α that become phosphorylated in response
33 to insulin or IGF-1 (Figs 2 and 5) and that
34 phosphorylation of both residues is required to
35 generate a high level of PKB α activity. Thus mutation
36 of either Thr-308 or Ser-473 to Ala greatly decreased

1 the activation of transfected PKB α by insulin or IGF-1
2 in 293 cells (Fig 8). Moreover, PKB α became partially
3 active in vitro when either Thr-308 or Ser-473 were
4 changed to Asp or when Ser-473 was phosphorylated by
5 MAPKAP kinase-2 in vitro, and far more active when the
6 D308 mutant of PKB α was phosphorylated by MAPKAP
7 kinase-2 or when Thr-308 and Ser-473 were both mutated
8 to Asp (Fig 12). Moreover, the D308/D473 double mutant
9 could not be activated further by stimulating cells
10 with insulin (Fig 13). These observations demonstrate
11 that the phosphorylation of Thr-308 and Ser-473 act
12 synergistically to generate a high level of PKB α
13 activity.

14
15 Thr-308, and the amino acid sequence surrounding it, is
16 conserved in rat PKB β and PKB γ but, interestingly,
17 Ser-473 (and the sequence surrounding it) is only
18 conserved in PKB β . In rat PKB γ , Ser-473 is missing
19 because the C-terminal 23 residues are deleted. This
20 suggests that the regulation of PKB γ may differ
21 significantly from that of PKB α and PKB β in the rat.

22
23 Thr-308 is located in subdomain VIII of the kinase
24 catalytic domain, nine residues upstream of the
25 conserved Ala-Pro-Glu motif, the same position as
26 activating phosphorylation sites found in many other
27 protein kinases. However, Ser-473 is located C-terminal
28 to the catalytic domain in the consensus sequence
29 Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr which is present in
30 several protein kinases that participate in growth
31 factor-stimulated kinase cascades, such as p70 S6
32 kinase, PKC and p90rsk (Pearson et al, 1995). However,
33 it is unlikely that a common protein kinase
34 phosphorylates this motif in every enzyme for the
35 following reasons. Firstly, phosphorylation of the
36 equivalent site in p70 S6 kinase is prevented by the

1 immunosuppressant drug rapamycin (Pearson et al, 1995)
2 which does not prevent the activation of PKB α by
3 insulin (Cross et al, 1995) or is phosphorylation at
4 Ser-473 (D. Alessi, unpublished work). Secondly, the
5 equivalent residue in protein kinase C is
6 phosphorylated constitutively and not triggered by
7 stimulation with growth factors (Tsutakawa et al.,
8 1995).

9
10 MAPKAP kinase-2 is a component of a protein kinase
11 cascade which becomes activated when cells are
12 stimulated with interleukin-1 or tumour necrosis factor
13 or exposed cellular stresses (Rouse et al, 1994; Cuenda
14 et al, 1995). MAPKAP kinase-2 phosphorylates PKB α
15 stoichiometrically at Ser-473 (Fig 11) and this finding
16 was useful in establishing the role of Ser473
17 phosphorylation in regulating PKB α activity. However,
18 although MAPKAP kinase-2 activity is stimulated to a
19 small extent by insulin in L6 cells, no activation
20 could be detected in 293 cells in response to insulin
21 or IGF-1. Moreover, exposure of L6 cells or 293 cells
22 to a chemical stress (0.5 mM sodium arsenite) strongly
23 activated MAPKAP kinase-2 (D. Alessi, unpublished work)
24 as found in other cells (Rouse et al, 1994; Cuenda et
25 al, 1995), but did not activate PKB α at all.
26 Furthermore, the drug SB 203580 which is a specific
27 inhibitor of the protein kinase positioned immediately
28 upstream of MAPKAP kinase-2 (Cuenda et al, 1995),
29 prevented the activation of MAPKAP kinase-2 by arsenite
30 but had no effect on the activation of PKB α by insulin
31 or IGF-1. Finally, the activation of PKB α was prevented
32 by wortmannin (Figs 6 and 9), but wortmannin had no
33 effect on the activation of MAPKAP kinase-2 in L6 or
34 293 cells. It should also be noted that the sequence
35 surrounding Ser-473 of PKB α (HFPQFSY) does not conform
36 to the optimal consensus for phosphorylation by MAPKAP

1 kinase-2 which requires Arg at position n-3 and a bulky
2 hydrophobic residue at position n-5, (where n is the
3 position of the phosphorylated residue). The K_m for
4 phosphorylation of the synthetic peptide comprising
5 residues 465-478 is nearly 100-fold higher than the K_m
6 for the standard MAPKAP kinase-2 substrate peptide
7 (data not shown). It is therefore unlikely that MAPKAP
8 kinase-2 mediates the phosphorylation of Ser-473 in
9 vivo.

10

11 The enzyme(s) which phosphorylates Thr-308 and Ser-473
12 in vivo does not appear to be PKB α itself. Thus
13 incubation of the partially active Asp-308 mutant with
14 MgATP did not result in the phosphorylation of Ser-473,
15 phosphorylation of the latter residue only occurring
16 when MAPKAP kinase-2 was added (Fig 11A, Fig 12).
17 Similarly, Thr-308 did not become phosphorylated when
18 either the partially active D473 mutant or the
19 partially active Ser-473 phosphorylated form of PKB α
20 were incubated with MgATP. PKB α when bound to lipid
21 vesicles containing phosphatidylserine and PIP3 also
22 fails to activate upon incubation with MgATP (Alessi et
23 al, 1996) and after transfection into 293 cells, a
24 "kinase dead" mutant of PKB α became phosphorylated on
25 Thr-308 and Ser-473 in response to insulin or IGF-1
26 (Fig 14). Furthermore, HA-PKB α from either unstimulated
27 or insulin-stimulated 293 cells failed to phosphorylate
28 the synthetic C-terminal peptide comprising amino acids
29 467-480.

30

31 In unstimulated L6 myotubes, the endogenous PKB α was
32 phosphorylated at a low level at a number of sites (Fig
33 6A), but in unstimulated 293 cells the transfected
34 enzyme was heavily phosphorylated at Ser-124 and
35 Thr-450 (Fig 10). Ser-124 and Thr-450 are both followed
36 by proline residues suggesting the involvement of

1 "proline-directed" protein kinases. Although, the
2 phosphorylation of Ser-124 was greatly decreased when
3 "kinase dead" PKB α was transfected into 293 cells (Fig
4 14), it would be surprising if Ser-124 is
5 phosphorylated by PKB α itself because the presence of a
6 C-terminal proline abolishes the phosphorylation of
7 synthetic peptides by PKB α (D.Alessi, unpublished
8 work). Since transfected PKB α is inactive in
9 unstimulated 293 cells (Fig 12), phosphorylation of
10 Ser-124 and Thr-450 clearly does not activate PKB α
11 directly. Ser-124 is located in the linker region
12 between the PH domain and the catalytic domain of the
13 mammalian PKB α isoforms but, unlike Thr-450, is not
14 conserved in the Drosophila homologue (Andjelkovic et
15 al, 1995).

16
17 While we do not wish to be bound by hypotheses, the
18 results described suggest that agonists which activate
19 PI 3-kinase are likely to stimulate PKB α activity via
20 one of the following mechanisms. Firstly, PIP3 or
21 PI3,4-bisP may activate one or more protein kinases
22 which then phosphorylate PKB α at Thr-308 and Ser-473.
23 Secondly, the formation of PIP3 may lead to the
24 recruitment of PKB α to the plasma membrane where it is
25 activated by a membrane-associated protein kinase(s).
26 The membrane associated Thr-308 and Ser-473 kinases
27 might themselves be activated by PIP3 and the
28 possibility that Thr-308 and/or Ser-473 are
29 phosphorylated directly by PI 3-kinase has also not
30 been excluded, because this enzyme is known to
31 phosphorylate itself (Dhand et al, 1994) and other
32 proteins (Lam et al, 1994) on serine residues.

33

34 **Example 8: Molecular basis for substrate specificity of**
35 **PKB.** PKB α has been shown to influence GSK3 activity.
36 GSK3 α and GSK3 β are phosphorylated at Ser-21 and Ser-9,

1 respectively, by two other insulin-stimulated protein
2 kinases, namely p70 S6 kinase and MAP kinase-activated
3 protein kinase-1 (MAPKAP-K1, also known as p90 S6
4 kinase). However, these enzymes are not rate-limiting
5 for the inhibition of GSK3 by insulin in L6 myotubes
6 because specific inhibitors of their activation
7 (rapamycin-p70 S6 kinase; PD 98059-MAPKAP kinase-1)
8 have no effect (Cross et al., 1995).

9
10 The activation of PI 3-kinase is essential for many of
11 the effects of insulin and growth factors, including
12 the stimulation of glucose transport, fatty acid
13 synthesis and DNA synthesis, protection of cells
14 against apoptosis and actin cytoskeletal rearrangements
15 (reviewed in Carpenter et al., 1996). These
16 observations raise the question of whether PKB α
17 mediates any of these events by phosphorylating other
18 proteins. To address this issue we characterised the
19 substrate specificity requirements of PKB α . We find
20 that the optimal consensus sequence for phosphorylation
21 by PKB α is the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd,
22 where Yaa and Zaa are small amino acids (other than
23 glycine) and Hyd is a large hydrophobic residue (such
24 as Phe or Leu). We also demonstrate that PKB α
25 phosphorylates histone H2B (a substrate frequently used
26 to assay PKB α in vitro) at Ser-36 which lies in an Arg-
27 Xaa-Arg-Xaa-Xaa-Ser-Hyd motif. These studies identified
28 a further PKB α substrate (Arg-Pro-Arg-Ala-Ala-Thr-Phe)
29 that, unlike other peptides, is not phosphorylated to a
30 significant extent by either p70 S6 kinase or MAPKAP-
31 K1.

34 Results

35 Preparation of Protein Kinase B α

36 In order to examine the substrate specificity of PKB α ,

1 it was first necessary to obtain a kinase preparation
2 that was not contaminated with any other protein kinase
3 activities. 293 cells were therefore transiently
4 transfected with a DNA construct expressing
5 haemagglutinin-tagged PKB α (HA-PKB α), stimulated with
6 IGF-1 and the HA-PKB α immunoprecipitated from the
7 lysates). IGF-1 stimulation resulted in a 38-fold
8 activation of PKB α (Fig 16) and analysis of the
9 immunoprecipitates by SDS-polyacrylamide gel
10 electrophoresis revealed that the 60 kDa PKB α was the
11 major protein staining with Coomassie Blue apart from
12 the heavy and light chains of the haemagglutinin
13 monoclonal antibody (Fig 16, Lanes 2 and 3). The minor
14 contaminants were present in control immunoprecipitates
15 derived from 293 cells transfected with an empty pCMV5
16 vector but lacked HA-PKB activity (Fig 16, Lane 4).
17 Furthermore, a catalytically inactive mutant HA-
18 PKB α immunoprecipitated from the lysates of IGF-1
19 stimulated 293 cells had no Crosstide kinase activity
20 (Alessi et al., 1996). Thus, all the Crosstide activity
21 in HA-PKB immunoprecipitates is catalysed by PKB α .

22

23 Identification of the residues in histone H2B

24 phosphorylated by PKB α . Currently, three substrates are
25 used to assay PKB α activity in different laboratories,
26 histone H2B, MBP and Crosstide. PKB α phosphorylated
27 Crosstide with a K_m of 4 μ M and a V_{max} of 260 U/mg
28 (Table 7.1 A, peptide 1), histone H2B with a K_m of 5 μ M
29 and a V_{max} of 68 U/mg, and MBP with a K_m of 5 μ M and a
30 V_{max} of 25 U/mg. Thus the V_{max} of histone H2B and MBP
31 are 4-fold and 10-fold lower than for Crosstide. In
32 order to identify the residue(s) in histone H2B
33 phosphorylated by PKB α , 32 P-labelled histone H2B was
34 digested with trypsin (see Methods) and the resulting
35 peptides chromatographed on a C18 column at pH 1.9.
36 Only one major 32 P-labelled peptide (termed T1) eluting

1 at 19.5 % acetonitrile was observed (Fig 17A), The
2 peptide contained phosphoserine (data not shown), its
3 sequence commenced at residue 34 of histone H2B and a
4 single burst of radioactivity occurred after the third
5 cycle of Edman degradation (Fig 17B), demonstrating
6 that PKB α phosphorylates histone H2B at Ser-36 within
7 the sequence Arg-Ser-Arg-Lys-Glu-Ser-Tyr. Thus, like
8 the serine phosphorylated in Crosstide, Ser-36 of
9 histone H2B lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd
10 motif (where Hyd is a bulky hydrophobic residue -Phe in
11 Crosstide, Tyr in H2B).

12

13 Molecular basis for the substrate specificity of PKB α

14 To further characterise the substrate specificity
15 requirements for PKB α , we first determined the minimum
16 sequence phosphorylated efficiently by PKB α by removing
17 residues sequently from the C-terminal and N-terminal
18 end of Crosstide. Removal of the N-terminal glycine and
19 up to three residues from the C-terminus had little
20 effect on the kinetics of phosphorylation by PKB α
21 (Table 7.1A, compare peptides 1 and 5). However any
22 further truncation of either the N or C-terminus
23 virtually abolished phosphorylation (Table 7.1A,
24 peptides 8 and 9). The minimum peptide phosphorylated
25 efficiently by PKB α (Arg-Pro-Arg-Thr-Ser-Ser-Phe) was
26 found to be phosphorylated exclusively at the second
27 serine residue as expected. Consistent with this
28 finding, a peptide in which this serine was changed to
29 alanine was not phosphorylated by PKB α (Table 7.1A,
30 peptide 7). All further studies were therefore carried
31 out using variants of peptide 5 in Table 7.1A (see
32 below).

33

34 A peptide in which the second serine of peptide 5
35 (Table 7.1A) was replaced by threonine was
36 phosphorylated with a K_m of 30 μM and an unchanged V_{max}

(Table 7.1, peptide 6). All the ^{32}P -radioactivity incorporated was present as phosphothreonine and solid phase sequencing revealed that the peptide was only phosphorylated at the second threonine residue, as expected (data not shown). Thus PKB α is capable of phosphorylating threonine as well as serine residues, but has a preference for serine.

We next changed either of the two arginine residues in peptide 5 to lysine. These substitutions drastically decreased the rate of phosphorylation by PKB α (Table 7.1A, peptides 10 and 11) demonstrating a requirement for arginine (and not simply any basic residue) at both positions.

We then examined the effect of substituting the residues situated immediately C-terminal to the phosphorylated serine in peptide 5 (Table 7.1B). The data clearly demonstrate that the presence of a large hydrophobic residue at this position is critical for efficient phosphorylation, with the K_m increasing progressively with decreasing hydrophobicity of the residue at this position (Table 7.1B, peptides 1 to 4). Replacement of the C-terminal residue with Lys increased the K_m 18-fold and a substitution at this position with either Glu or Pro almost abolished phosphorylation (Table 7.1B, peptides 5-7).

Replacement of the Thr situated two residues N-terminal to the phosphorylated serine increased the K_m with any amino acid tested (Table 7.1C). Substitution with Ala only increased K_m by 2-3 fold, but substitution with other residues was more deleterious and with Asn (a residue of similar size and hydrophilicity to Thr) phosphorylation was almost abolished (Table 7.1C).

Replacement of the Ser situated one residue N-terminal

1 to the phosphorylated serine also increased the K_m with
2 any amino acid tested, but the effects were less severe
3 than at position n-2 (Table 7.1C). When residues n-2
4 and n-1 were both changed to Ala, the resulting peptide
5 RPRAASF was phosphorylated by PKB α with a K_m only 5-
6 fold higher than RPRTSSF. In contrast the peptides
7 RPRGGSF, RPRAGSF, and RPRGASF were phosphorylated less
8 efficiently (Table 7.1C).

9
10 Comparison of the substrate specificity of PKB α with
11 MAPKAP kinase-1, and p70 S6 kinase. Since MAPKAP-K1
12 and p70 S6 kinase phosphorylate the same residue in
13 GSK3 phosphorylated by PKB α , and studies with synthetic
14 peptides have established that MAPKAP-K1 and p70 S6
15 kinase also preferentially phosphorylate peptides in
16 which basic residues are present at positions n-3 and
17 n-5 (Leighton et al., 1995), we compared the
18 specificities of MAPKAP-K1, p70 S6 kinase and PKB α in
19 greater detail.

20
21 MAPKAP kinase-1 and p70 S6 kinase phosphorylate the
22 peptides KKKNRTLSVA and KKRNRRTLSVA with extremely low
23 K_m values of 0.2- 3.3 μM , respectively (Table 7.2).
24 However, these peptides were phosphorylated by PKB α
25 with 50-900 fold higher K_m values (Table 7.2A, peptides
26 1 and 2). The peptide KKRNRRTLTV, which is a relatively
27 specific substrate for p70 S6 kinase (Leighton et al.,
28 1995) was also phosphorylated very poorly by PKB α
29 (Table 7.2A, peptide 4).

30
31 Crosstide is phosphorylated by p70 S6 kinase and MAPKAP
32 kinase-1 with similar efficiency to PKB α ((Leighton et
33 al., 1995); Table 7.2B-peptide 1 and Fig 18). However,
34 truncation of Crosstide to generate the peptide RPRTSSF
35 was deleterious for phosphorylation by MAPKAP-K1 and
36 even worse for p70 S6 kinase (Table 7.2B-peptides 1 and

1 2 and Fig 18). Moreover, changing the phosphorylated
2 serine in RPRTSSF to threonine increased the K_m for
3 phosphorylation by p70 S6 kinase much more than for
4 PKB α and almost abolished phosphorylation by MAPKAP-K1
5 (Table 7.2B-peptide 3 and Fig 18). The peptide RPRAASF
6 was phosphorylated by MAPKAP-K1 with essentially
7 identical kinetics to that of PKB α ; however
8 phosphorylation by p70 S6 kinase was virtually
9 abolished (Table 7.2B-peptide 4 and Fig 18). Based on
10 these observations we synthesized the peptide RPRAATF.
11 This peptide was phosphorylated by PKB α with a K_m of
12 25 μ M and similar V_{max} to RPRTSSF, but was not
13 phosphorylated to a significant extent by either
14 MAPKAP-K1 or p70 S6 kinase (Table 7.2B-peptide 5, Fig
15 18). In Fig 18, the protein kinase concentration in
16 the assays towards Crosstide were 0.2 U/ml, and each
17 peptide substrate was assayed at a concentration of 30
18 μ M. Filled bars denote PKB α activity, hatched bars
19 MAPKAP kinase-1 activity, and grey bars p70 S6 kinase
20 activity. The activities of each protein kinase are
21 given relative to their activity towards Crosstide
22 (100). The results are shown \pm SEM for two experiments
23 each carried out in triplicate.

24

25 Discussion.

26 We have established that the minimum consensus sequence
27 for efficient phosphorylation by PKB α is Arg-Xaa-Arg-
28 Yaa-Zaa-Ser-Hyd, where Xaa is any amino acid, Yaa and
29 Zaa are small amino acid other than glycine (Ser, Thr,
30 Ala) and Hyd is a bulky hydrophobic residue (Phe, Leu)
31 (Table 7.1). The heptapeptide with the lowest K_m value
32 was RPRTSSF, its K_m of 5 μ M being comparable to many of
33 the best peptide substrates identified for other
34 protein kinases. The V_{max} for this peptide (250 nmoles
35 min⁻¹ mg⁻¹) may be an underestimate because the PKB α
36 was obtained by immunoprecipitation from extracts of

1 IGF-1 stimulated 293 cells over-expressing this protein
2 kinase, and a significant proportion of the PKB α may
3 not have been activated by IGF-1 treatment.

4
5 The requirement for arginine residues at positions n-3
6 and n-5 (where n is the site of phosphorylation) seems
7 important, because substituting either residue with
8 lysine decreases phosphorylation drastically. Serine
9 and threonine residues were preferred at positions n-1
10 and n-2, although the K_m value was only increased about
11 5-fold if both of these residues were changed to Ala.
12 Serine was preferred at position n, since changing it
13 to threonine caused a six-fold increase in the K_m .
14 The peptide RPRAATF, which was phosphorylated with a K_m
15 of 25 μ M and similar V_{max} to RPRTSSF, may therefore be
16 a better substrate for assaying PKB α in partially
17 purified preparations, because unlike Crosstide, it
18 contains only one phosphorylatable residue and is not
19 phosphorylated significantly by MAPKAP-K1 or p70 S6
20 kinase (Table 7.2, Fig 18 and see below).

21
22 The Proline at position n-4 was not altered in this
23 study because it was already clear that this residue
24 was not critical for the specificity of PKB α . Residue
25 n-4 is proline in GSK3 β but alanine in GSK3 α . Both GSK3
26 isoforms are equally good substrates for PKB α in vitro
27 (Cross et al., 1995), and the peptide
28 GRARTSSFA (corresponding to the sequence in GSK3 α) is
29 phosphorylated by PKB α with a K_m of 10 μ M and V_{max} of
30 230 U/mg (Table 7.1A, peptide 2). Moreover, in histone
31 H2B, the residue located four amino acids N-terminal to
32 the PKB α phosphorylation site is serine (Fig 17). The
33 presence of Glu and Lys at positions n-1 and n-2 may
34 explain why histone H2B is phosphorylated by PKB α with
35 a four-fold lower V_{max} than the peptide RPRTSSF.

36

1 Two other protein kinases which are activated by
2 insulin and other growth factors, p70 S6 kinase and
3 MAPKAP-K1, require basic residues at positions n-3 and
4 n-5 (Leighton et al., 1995), explaining why they also
5 phosphorylate and inactivate GSK3 in vitro (Sutherland
6 et al., 1993). Indeed, there is evidence that MAPKAP-
7 K1 plays a role in the inhibition of GSK3 by EGF
8 because, unlike inhibition by insulin which is
9 prevented by inhibitors of PI 3-kinase, the inhibition
10 of GSK3 by EGF is only suppressed partially by
11 inhibitors of PI 3-kinase. Moreover, in NIH 3T3 cells,
12 the inhibition of GSK3 α and GSK3 β by EGF is largely
13 prevented by expression of a dominant negative mutant
14 of MAP kinase kinase-1 (Eldar et al., 1995). In
15 contrast, p70 S6 kinase is not rate limiting for the
16 inhibition of GSK3 in the cells that have been examined
17 so far because rapamycin, which prevents the activation
18 of p70 S6 kinase by EGF or insulin, has no effect on
19 the inhibition of GSK3 by these agonists (Cross et al.,
20 1995 and Saito et al., 1994).

21

22 Additional similarities between p70 S6 kinase, MAPKAP-
23 K1 and PKB α include the failure to phosphorylate
24 peptides containing Pro at position n+1 and dislike of
25 a lysine at the same position. This suggests that, in
26 vivo, these kinases are unlikely to phosphorylate the
27 same residues as MAP kinases (which phosphorylates
28 Ser/Thr-Pro motifs) or protein kinase C (which prefers
29 basic residues C-terminal to the site of
30 phosphorylation). However, the present work has also
31 revealed significant differences in the specificities
32 of these enzymes. In particular MAPKAP-K1 and (to a
33 lesser extent) p70 S6 kinase can tolerate substitution
34 of the Arg at position n-5 by lysine whereas PKB α
35 cannot (see Table 7.1A, Table 7.2A and (Leighton et
36 al., 1995)). MAPKAP-K1 and p70 S6 kinase can also

1 tolerate, to some extent, substitution of Arg at
2 position n-3 by Lys. For example, the peptide
3 KKRNKTLSVA is phosphorylated by MAPKAP-K1 and p70 S6
4 kinase with K_m values of 17 and 34 μM , respectively,
5 as compared to K_m values of 0.7 and 1.5 μM for the
6 peptide KKRNRTLSVA (Table 7.2A). In contrast, PKB α
7 does not phosphorylate the peptide KKRNKTLSVA (Table
8 7.2A) or any other peptide that lacks Arg at position
9 n-3. PKB α and p70 S6 kinase, but not MAPKAP-K1,
10 phosphorylate Thr as well as Ser (Table 7.1A) and can
11 phosphorylate peptides lacking any residue at position
12 n+2 ((Leighton et al., 1995) and Table 7.2A), while
13 PKB α and MAPKAP-K1, but not p70 S6 kinase, can tolerate
14 substitution of both the n-1 and n-2 positions of the
15 peptide RPRTSSF with Ala (Table 7.2B). These
16 differences explain why the peptide RPRAATF is a
17 relatively specific substrate for PKB α .

18
19 One of the best peptide substrates for MAPKAP-K1 and
20 p70 S6 kinase (KKRNRTLSVA) was a poor substrate for
21 PKB α (Table 7.2, peptide 2), despite the presence of
22 Arg at positions n-3 and n-5. The presence of Leu at
23 position n-1 and Val at position n+1 are likely to
24 explain the high K_m for phosphorylation, because PKB α
25 prefers a small hydrophilic residue at the former
26 position and a larger hydrophobic residue at the latter
27 position (Tables 7.1 and 7.2).

28

29 **Example 9:**

30 This example demonstrates that coexpression of GSK3 in
31 293 cells with either the wild type or a constitutively
32 activated PKB results in GSK3 becoming phosphorylated
33 and inactivated. However coexpression of a mutant of
34 GSK3 in which Ser-9 is mutated to an Ala residue is not
35 inactivated under these conditions. These experiments
36 provide further evidence that PKB α activation can

1 mediate the phosphorylation and inactivation of GSK3 in
2 a cellular environment, and could be used as an assay
3 system to search for specific PKB inhibitors.

4

5 Monoclonal antibodies recognising the sequence EFMPME
6 (EE) antibodies and the (EQKLISEEDL) c-Myc purchased
7 from Boehringer (Lewis, UK).

8

9 **Construction of expression vectors and transfections**
10 **into 293 cells.** HA-PKBa, HA-KD-PKB and 308D/473D
11 HA-PKBa was described previously (Alessi et al., 1996).

12

13 A DNA construct expressing human GSK3B with the EFMPME
14 (EE) epitope tag at the N-terminus was prepared as
15 follows: A standard PCR reaction was carried out using
16 as a template the human GSK3 β cDNA clone in the
17 pBluescript SK+ vector and the oligonucleotides

18

19 GCGGAGATCTGCCACCATGGAGTTCATGCCCATGGAGTCAGGGCGGCCCAACC

20

21 and GCGGTCCGGAACATAGTCCAGCACCAG that incorporate a *bgl*
22 II site (underlined) and a *Bspe* I site (double
23 underlined). A three-way ligation was then set up in
24 which the resulting PCR product was subcloned as a *Bgl*
25 II-*Bspe* I fragment together with the C-terminal *Bspe*
26 I-Cla I fragment of GSK3 β into the *Bgl* II-Cla I sites
27 of the pCMV5 vector (Anderson et al., 1989). The
28 construct was verified by DNA sequencing and purified
29 using the Quiagen plasmid Mega kit according to the
30 manufacturers protocol. The c-Myc GSK3, BA9 construct
31 encodes GSK3 β in which Ser-9 is mutated to Ala and
32 possesses a c-myc epitope tag at the C-terminus and was
33 prepared as described in Sperber et al., 1995. The
34 c-Myc GSK3 β A9 gene was then subcloned into *xba* I/*ECOR*
35 I sites of the pCMV5 eukaryotic expression vector.

36

1 **Cotransfection of GSK3 β with PKBa and its assay.**
2 293 cells growing on 10 cm diameter dishes were
3 transfected with 10 ug of DNA constructs expressing
4 EE-GSK3, Myc-GSK3A9 in the presence or absence of
5 HA-PKB, HA-KD-PKB or HA-308D/473D-PKB exactly as
6 described in Alessi et al., 1996. The cells were grown
7 in the absence of serum for 16 h prior to lysis, and
8 then lysed in 1.0 ml of ice-cold Buffer A (50 mM
9 Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton
10 X100, 1 mM sodium orthovanadate, 10 mM sodium
11 glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate,
12 1 μ M Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine,
13 0.2 mM phenylmethylsulphonyl fluoride, 10 ug/ml
14 leupeptin, and 0.1% (by vol) 2-mercaptoethanol). The
15 lysate was centrifuged at 4°C for 10 min at 13, 000 x g
16 and an aliquot of the supernatant (100 ug protein) was
17 incubated for 30 min on a shaking platform with 5 ul of
18 protein G-Sepharose coupled to 1ug of EE monoclonal
19 antibody. The suspension was centrifuged for 1min at
20 13,000 x g, the Protein G-Sepharose-antibody-EE-GSK3 β
21 complex washed twice with 1.0 ml of Buffer A containing
22 0.5 M NaCl, and three times with Buffer B (50 mM Tris
23 pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1%
24 (by vol) 2-mercaptoethanol), and the immunoprecipitate
25 assayed for GSK3 activity after incubation with either
26 PP2A or microcystin inactivated PP2A as described
27 previously (Cross et al., 1994).

28

29 **Results**

30

31 **Cotransfection of GSK3 β with PKBa in 293 cells results**
32 **in GSK3 phosphorylation and inactivation**
33 Human embryonic kidney 293 cells were transfected with
34 a DNA construct expressing EE-epitope tagged GSK3 β
35 either in the presence or absence of DNA constructs
36 expressing wild type-PKBa, a catalytically inactive

1 PKBa or a constitutively active HA-(308D/473D)-PKBa.
2 Cells were serum starved for 16 h. 36h post
3 transfection the cells were lysed, and the GSK3 β
4 immunoprecipitated from the lysates using monoclonal EE
5 antibodies and the GSK3 β activity measured before and
6 after treatment with PP2A. When EEGSK3 β was expressed
7 alone or in the presence of a catalytically inactive
8 PKBa, treatment of the EE-GSK3 β with PP2A only resulted
9 in about a 12% increase in activity (Fig 19A). However
10 when EE-GSK3 β was coexpressed with either the wild type
11 PKBa or the constitutively activated 308D/473D-HA-PKBa,
12 treatment of the EE-GSK3 from these cell lysates with
13 PP2A resulted in a 68% and 85% increase in the GSK3
14 activity, respectively. Coexpression of Myc-GSK3 β A9
15 with HA-PKB or the constitutively active
16 308D/473D-HA-PKBa did not result in any significant
17 inactivation of this mutant of GSK3 as judged by its
18 ability to be reactivated by PP2A (Fig 19B). These data
19 demonstrate that even in a cellular environment, PKBa
20 is capable of phosphorylating GSK3 β at Ser-9 and
21 inactivation of the enzyme. To estimate the relative
22 levels of EE-GSK3 β and PKBa, EE-GSK3 and HA-PKBa were
23 immunoprecipitated from equal volumes of cell lysate,
24 and the immunoprecipitates run on an SDS-polyacrylamide
25 gel, and the gel stained with Coomassie Blue. These
26 experiments revealed that both the wild type HA-PKBa
27 and the 308D/473D-PKBa were expressed at a 20 to 30
28 -fold higher level than GSK3a, whereas KD-PKBa is
29 expressed at a level that is about 5-fold lower than
30 that of the wild type PKBa. Under the conditions used
31 for the immunoprecipitations, no PKBa was
32 co-immunoprecipitated with GSK3 β , or no GSK3 β was
33 co-immunoprecipitated with the PKBa (data not shown).
34 Coexpression of EE-GSK3 β with all forms of PKBa
35 resulted in about a 2-3 fold decrease in the level of
36 expression on EE-GSK3 β compared to when it is expressed

1 alone in cells.

2

3 Example 10: basic assay for identifying agents which
4 affect the activity of PKB.

5 A 40 μ l assay mix was prepared containing protein
6 kinase (0.2U/ml) in 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA,
7 0.1% (by vol) 2-mercaptoethanol, 2.5 μ M PKI, protein
8 kinase substrate (30 μ M), and the indicated
9 concentration of Ro-318220 or GC 109203X (test
10 inhibitors). After incubation on ice for 10 min the
11 reaction was started by the addition of 10 μ l of 50mM
12 magnesium acetate and 0.5 mM [γ ³²P]ATP (100-200
13 cpm/pmol). For the assay of mixed isoforms of PKC 20
14 μ M diacylglycerol, 0.5 mM CaCl₂, and 100 μ M
15 phosphatidylserine were also present in the
16 incubations. The assays were carried out for 15 min at
17 30°C, then terminated and analysed as described (Alessi
18 1995). One unit of activity was that amount of enzyme
19 that catalysed the phosphorylation of 1nmol os
20 substrate in 1 min. The final concentration of DMSO in
21 each assay was 1% (by vol). This concentration of DMSO
22 does not inhibit any of these enzymes. Mixed isoforms
23 of PKC were assayed using histone H1 as substrate,
24 while MAPKAP-K1 β and p70 S6 kinase were assayed using
25 the peptide KKRNRTLSVA (Leighton 1995). Protein kinase
26 B was assayed with the peptide GRPRTSSFAEG [9] and
27 MAPKAP-K2 was assayed with the peptide KKLNRTLSVA
28 (Stokoe 1993). p42 MAP kinase was assayed using MBP,
29 and MAPKK-1, and c-Raf1 were assayed as described in
30 Alessi 1995.

31

32 **Results**

33 Effect of Ro 318220 and GF 109203X on protein kinases
34 activated by growth factors, cytokines and cellular
35 stresses. The mixed isoforms of PKC were potently
36 inhibited by Ro 318220, with an IC₅₀ of 5 nM in our

1 assay (Fig 20A). In contrast, a number of protein
2 kinases activated by growth factors (c-Raf1, MAPKK-1,
3 p42 MAP kinase) and one protein kinase that is
4 activated by cellular stresses and proinflammatory
5 cytokines (MAPKAP-K2) were not inhibited significantly
6 by Ro 318022 in vitro (Fig 20A). Protein kinase B, an
7 enzyme that is activated in response to insulin and
8 growth factors was inhibited by Ro 318220 (IC_{50} of 1 μ M,
9 Fig 20B) similar to the IC_{50} for PKA. However, to our
10 surprise, MAPKAP-K1B an enzyme which lies immediately
11 downstream of p42 and p44 MAP kinases and which is
12 activated in response to every agonist that stimulates
13 this pathway, was inhibited by Ro 318220 even more
14 potently than the mixed PKC isoforms (IC_{50} = 3nM, Fig
15 20B). The p70 S6 kinase, which lies on a distinct
16 growth factor-stimulated signalling pathway from
17 MAPKAP-K1B, was also potently inhibited by Ro 318220
18 (IC_{50} =15 nM, Fig 20B).

19

20 Similar results were obtained using GF 109203X instead
21 of Ro 3318220. As reported previously (Toullec et al.,
22 1991), GF 109203X inhibited the mixed isoforms of PKC
23 (IC_{50} =30 nM) without inhibiting protein kinase B (Fig
24 21) or c-Raf, MAPKK-1 and p42 MAP kinase (data not
25 shown). However MAPKAP-K1B and p70 S6 kinase were
26 potently inhibited by this compound with IC_{50} values of
27 50 nM and 100 nM, respectively (Fig 21).

28

29

1 **General Materials and Methods** Tissue culture reagents, myelin
2 basic protein (MBP), microcystin-LR, and IGF-1 were obtained from
3 Life Technologies Inc. (Paisley, UK), insulin from Novo-Nordisk
4 (Bagsvaerd, Denmark), phosphate free Dulbecco's minimal essential
5 medium (DMEM) from (ICN, Oxon, UK), Protein G-Sepharose and
6 CH-Sepharose from Pharmacia (Milton Keynes, UK), alkylated trypsin
7 from Promega (Southampton, UK), 4-vinylpyridine, wortmannin and
8 fluoroisothiocyanate-labelled antimouse IgG from goat from
9 Sigma-Aldrich (Poole, Dorset, UK). Polyclonal antibodies were
10 raised in sheep against the peptides RPHFPQFSYSASGTA
11 (corresponding to the last 15 residues of rodent PKB α) and
12 MTSALATMRVDYEQIK (corresponding to residues 352 to 367 of human
13 MAPKAPkinase-2) and affinity purified on peptide-CH-Sepharose.
14 Monoclonal HA antibodies were purified from the tissue culture
15 medium of 12CA5 hybridoma and purified by chromatography on
16 Protein G-Sepharose. The peptide RPRHFPQFSYSAS, corresponding to
17 residues 465-478 of PKB α , was synthesized on an Applied Biosystems
18 430A peptide synthesizer. cDNA encoding residues 46-400 of human
19 MAPKAP kinase-2 was expressed in E.coli as a glutathione
20 S-transferase fusion protein and activated with p38/RK MAP KINASE
21 by Mr A.Clifton (University of Dundee) as described previously
22 (Ben-Levy et al., 1995).
23
24 Monoclonal antibodies recognising the haemagglutinin (HA) epitope
25 sequence YPYDVPDYA, Protein G-Sepharose and histone H2B were
26 obtained from Boehringer (Lewes, UK). MAPKAP kinase-1 (Sutherland
27 et al., 1993) and p70 S6 kinases (Leighton et al., 1995) were
28 purified from rabbit skeletal muscle and rat liver respectively.
29
30 **Construction of expression vectors.** The pECE constructs encoding
31 the human HAPKB α and kinase-dead (K179A) HA-KD-PKB α have already
32 been described (Andjelkovic et al., 1996). The mutants at Ser-473
33 (HA-473A PKB α and HA-473D PKB α) were created by PCR using a 5'
34 oligonucleotide encoding amino acids 406 - 414 and mutating 3'
35 oligonucleotide encoding amino acids 468 - 480, and the resulting
36 PCR products subcloned as *CelII*-*EcoRI* fragment into pECE.HA-PKB α .
37 The Thr-308 mutants (HA-308A PKB α and HA308D PKB α) were created by
38 the two-stage PCR technique (No et al., 1989) and subcloned as
39 *NotI*-*EcoRI* fragments into pECE.HA-PKB. The double mutant
40 HA-308D/473D PKB was made by subcloning the *CelII*-*EcoRI* fragment
41 encoding 473D into pECE.HA-308D PKB α . For construction of
42 cytomegalovirus-driven expression constructs, *BglIII*-*XbaI* fragments
43 from the appropriate pECE constructs were subcloned into the same
44 restriction sites of the pCMV5 vector (Andersson et al., 1989).

1 All constructs were confirmed by restriction analysis and
2 sequencing and purified using Quiagen Plasmid Maxi Kit according
3 to the manufacturer's protocol. All oligonucleotide sequences are
4 available upon request.

5
6 **³²P-labelling of L6 myotubes and immunoprecipitation of PKB α .** L6
7 cells were differentiated into myotubes on 10 cm diameter dishes
8 (Hundal et al., 1992). The myotubes were deprived of serum
9 overnight in DMEM, washed three times in phosphate free DMEM and
10 incubated for a further 1 h with 5 ml of this medium. The myotubes
11 were then washed twice with phosphate free DMEM and incubated for
12 4 h with carrier-free [³²P]orthophosphate (1 mCi/ml). Following
13 incubation in the presence or absence of 100 nM wortmannin for 10
14 min, the myotubes were stimulated for 5 min at 37°C in the
15 presence or absence of 100 nM insulin and placed on ice. The
16 medium was aspirated, the myotubes washed twice with ice-cold DMEM
17 buffer and then lysed with 1.0 ml of ice-cold Buffer A (50 mM
18 Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X100, 1 mM
19 sodium orthopervanadate, 10 mM sodium glycerophosphate, 50 mM NaF,
20 5 mM sodium pyrophosphate, 1 μ M Microcystin-LR, 0.27 M sucrose, 1
21 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μ g/ml
22 leupeptin, and 0.1% (by vol) 2-mercaptoethanol). The lysates were
23 centrifuged at 4°C for 10 min at 13,000 x g and the supernatants
24 incubated for 30 min on a shaking platform with 50 μ l of Protein
25 G-Sepharose coupled to 50 μ g of preimmune sheep IgG. The
26 suspensions were centrifuged for 2 min at 13,000 x g and the
27 supernatants incubated for 60 min with 30 μ l of Protein G--
28 Sepharose covalently coupled to 60 μ g of PKB α antibody (Harlow and
29 Lane, 1988). The Protein G-Sepharose-antibody-PKB α complex was
30 washed eight times with 1.0 ml of Buffer A containing 0.5 M NaCl,
31 and twice with 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA and 0.1% (by
32 vol) 2-mercaptoethanol (Buffer B).

33
34 **Assay of immunoprecipitated PKB α and protein determinations.** Three
35 aliquots of each immunoprecipitate (each comprising only 5% of the
36 total immunoprecipitated PKB α) were assayed for PKB α activity
37 towards the peptide GRPRTSSFAEG as described previously (Cross et
38 al., 1995). One unit of activity was that amount which catalysed
39 the phosphorylation of 1 nmol of substrate in 1 min. Protein
40 concentrations were determined by the method of Bradford, 1976.

41
42 **Tryptic digestion of in vivo phosphorylated PKB α .** The
43 immunoprecipitated PKB α was added to an equal volume of 2% (by

1 mass) SDS and 2 % (by vol) 2-mercaptoethanol, and incubated for 5
2 min at 100°C. After cooling to room temperature, 4-vinylpyridine
3 was added to a final concentration of 2 % (by vol) and the mixture
4 was incubated for 1h at 30°C on a shaking platform, followed by
5 electrophoresis on a 10% polyacrylamide gel. After
6 autoradiography, the 60 kDa band corresponding to rat PKB α was
7 excised and the gel piece homogenized in five vols of 25 mM
8 N-ethylmorpholine HCl, pH 7.7, containing 0.1% (by mass) SDS and 5
9 % (by vol) 2-mercaptoethanol. The suspension was incubated for 1 h
10 at 37°C on a shaking platform, then centrifuged for 1 min at
11 13,000 x g and the supernatant collected. The pellet was incubated
12 for a further 1h with five vols of the same buffer and centrifuged
13 for 1min at 13,000 xg. The two supernatants (containing 80-90% of
14 the ³²P-radioactivity) were combined, 0.2 vols of 100% (by mass)
15 trichloroacetic acid added, and the sample incubated for 1 h on
16 ice. The suspension was centrifuged for 10 min at 13,000 x g, the
17 supernatant discarded and the pellet washed five times with 0.2 ml
18 of water. The pellet was then incubated at 30°C with 0.3 ml of 50
19 mM Tris/HCl pH 8.0, 0.1% (by vol) Triton X100 containing 1 μ g of
20 alkylated trypsin. After 3 h another 1 μ g of trypsin was added and
21 the suspension left for a further 12 h. Guanidinium hydrochloride
22 (8 M) was added to bring the final concentration to 1.0 M in order
23 to precipitate any residual SDS and, after standing on ice for 10
24 min, the suspension was centrifuged for 5 min at 13, 000 x g. The
25 supernatant containing 90 % of the ³²P-radioactivity was
26 chromatographed on a Vydac C18 column as described in the legend
27 to Fig 2.

28
29 **Transfection of 293 cells and immunoprecipitation of HA-tagged**
30 **PKB α .** Human embryonic kidney 293 cells were cultured at 37°C in
31 an atmosphere of 5% CO₂, on 10 cm diameter dishes in DMEM
32 containing 10 % foetal calf serum. Cells were split to a density
33 of 2 x 10⁶ per 10 cm dish, and after 24 h at 37°C the medium was
34 aspirated and 10 ml of freshly prepared DMEM containing 10 %
35 foetal calf serum added. Cells were transfected by a modified
36 calcium phosphate method (Chen and Okayama, 1988) with 1 μ g/ml DNA
37 per plate. 10 μ g of plasmid DNA in 0.45 ml of sterile water was
38 added to 50 μ l of sterile 2.5 M CaCl₂, and then 0.5 ml of a
39 sterile buffer composed of 50 mM N,N-bis(2-hydroxyethyl)-2-
40 aminoethanesulphonic acid/HCl pH 6.96, 0.28 M NaCl and 1.5 mM
41 Na₂HPO₄ was added. The resulting mixture was vortexed for 1 min,
42 allowed to stand at room temperature for 20 min, and then added
43 dropwise to a 10 cm dish of 293 cells). The cells were placed in

1 an atmosphere of 3% CO₂, for 16 h at 37°C, then the medium was
2 aspirated, and replaced with fresh DMEM containing 10% foetal calf
3 serum. The cells were incubated for 12 h at 37°C in an atmosphere
4 of 5% CO₂, and then for 12 h in DMEM in the absence of serum.
5 Cells were preincubated for 10 min in the presence of 0.1% DMSO or
6 100 nM wortmannin in 0.1% DMSO and then stimulated for 10 min with
7 either 100 nM insulin or 50 ng/ml IGF-1 in the continued presence
8 of wortmannin. After washing twice with ice cold DMEM the cells
9 were lysed in 1.0 ml of icecold Buffer A, the lysate was
10 centrifuged at 4°C for 10 min at 13,000 x g and an aliquot of the
11 supernatant (10 µg protein) was incubated for 60 min on a shaking
12 platform with 5 µl of protein G-Sepharose coupled to 2 µg of HA
13 monoclonal antibody. The suspension was centrifuged for 1 min at
14 13,000 x g, the Protein G-Sepharose-antibody-HA-PKBα complex
15 washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, and
16 twice with Buffer B, and the immunoprecipitate assayed for PKBα
17 activity as described above.

18
19 **³²P-Labeling of 293 cells transfected with HA-PKBα.** 293 cells
20 transfected with HA-PKBα DNA constructs. were washed with
21 phosphate free DMEM, incubated with [32p] orthophosphate (1
22 mCi/ml) as described for L6 myotubes, then stimulated with insulin
23 or IGF1 and lysed, and PKBα immunoprecipitated as described above.
24 The ³²P-labelled HA-PKBα immunoprecipitates were washed, alkylated
25 with 4-vinylpyridine, electrophoresed and digested with trypsin as
26 described above for the endogenous PKBα present in rat L6
27 myotubes.

28
29 **Transfection of COS-1 cells and immunoprecipitation of HA-PKBα.**
30 COS-1 cells were maintained in DMEM supplemented with 10% FCS at
31 37°C in an atmosphere of 5% CO₂. Cells at 70 - 80% confluency
32 were transfected by a DEAE-dextran method (Seed & Aruffo, 1987),
33 and 48 hours later serum-starved for 24 hours. Cells were lysed in
34 a buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Nonidet
35 P-40, 25 mM NaF, 40 mM sodium-β-glycerophosphate, 0.1 mM sodium
36 orthopervanadate, 1 mM EDTA, 1mM benzamidine, 1 mM
37 phenylmethylsulphonyl fluoride, and lysates centrifuged for 15 min
38 at 13,000 x g at 4°C. Supernatants were pre cleared once for 30
39 min at 4°C with 0.1 vols of 50% Sepharose 4B/25% Pansorbin
40 (Pharmacia and Calbiochem, respectively) and HA-PKBα
41 immunoprecipitated from 1 mg of extract using the 12CA5 antibody
42 coupled to Protein A Sepharose beads. Immunoprecipitates were
43 washed twice with lysis buffer containing 0.5 M NaCl and once with

1 lysis buffer.

2

3 **Immunoblotting and quantification of levels of PKB α expression.**

4 Cell extracts were resolved by 7.5% SDS-PAGE and transferred to
5 Immobilon membranes (Millipore). Filters were blocked for 30 min
6 in a blocking buffer containing 5% skimmed milk in 1x TBS, 1%
7 Triton X-100 and 0.5% Tween 20, followed by a 2h incubation with
8 the 12CA5 supernatant 1000-fold diluted in the same buffer. The
9 secondary antibody was alkaline conjugated anti-mouse Ig from goat
10 (Southern Biotechnology Associates, Inc), 1000-fold diluted in the
11 blocking buffer. Detection was performed using AP colour
12 development reagents from Bio-Rad according to the manufacturer's
13 instructions. Quantification of levels of PKB α expression was
14 achieved by chemiluminescence, using fluroisothiocyanate-labelled
15 antimouse IgG from goat as the secondary antibody and the Storm
16 840/860 and ImageQuant software from Molecular Dynamics.

17

18 All peptides used to assay PKB α , and TTYADFIASGRTGRRNAIHD (the
19 specific peptide inhibitor of cyclic AMP dependent protein kinase
20 - PKI) were synthesised on an Applied Biosystems 431A peptide
21 synthesizer. Their purity (> 95%) was established by HPLC and
22 electrospray mass spectrometry, and their concentrations were
23 determined by quantitative amino acid analysis.

24

25 **Preparation and assay of PKB α .** The construction of cytomegalovirus
26 vectors (pCMV5) of the human haemagglutinin epitope-tagged wild
27 type (HA-PKB α) was described previously (Alessi et al., 1996).
28 293 cells grown on 10 cm dishes were transfected with a DNA
29 construct expressing HA-PKB α using a modified calcium phosphate
30 procedure (Alessi et al., 1996). The cells were deprived of serum
31 for 16h prior to lysis and, where indicated, were stimulated for
32 10 min in the presence of 50 ng/ml IGF-1 to activate PKB α . The
33 cells were lysed in 1.0 ml ice-cold Buffer A (50 mM Tris/HCl pH
34 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium
35 orthovanadate, 10 mM sodium β -glycerophosphate, 50 mM NaF, 5 mM
36 sodium pyrophosphate, 1 μ M Microcystin-LR, 0.27 M sucrose, 1 mM
37 benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μ g/ml
38 leupeptin, and 0.1 % (by vol) 2-mercaptoethanol) the lysate
39 centrifuged at 4°C for 10 min at 13, 000 x g and the supernatant
40 obtained from one 10 cm dish of cells (2-3 mg protein) was
41 incubated for 60 min on a shaking platform with 20 μ l of protein
42 G-Sepharose coupled to 10 μ g of HA monoclonal antibody. The
43 suspension was centrifuged for 1 min at 13, 000 x g, the Protein

1 G-Sepharose-antibody-HA-PKB α complex washed twice with 1.0 ml of
2 Buffer A containing 0.5 M NaCl, and twice with Buffer B (50 mM
3 Tris/HCl pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1% (by
4 vol) 2-mercaptoethanol). The PKB α immunoprecipitates were diluted
5 in Buffer B to an activity of 2.0 U/ml towards the Crosstide
6 peptide GRPRTSSFAEG and 0.1 ml aliquots snap frozen in liquid
7 nitrogen and stored at -80 °C. No significant loss of PKB α
8 activity occurred upon thawing the PKB α immunoprecipitates or
9 during storage at -80 °C for up to 3 months. The standard
10 PKB α assay (50 μ l) contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA,
11 0.1% (by vol) 2-mercaptoethanol, 2.5 μ M PKI, 0.2 U/ml PKB α ,
12 Crosstide (30 μ M), 10 mM magnesium acetate and 0.1 mM [γ -³²P]ATP
13 (100-200 cpm/pmol). The assays were carried out for 15 min at
14 30 °C, the assay tubes being agitated continuously to keep the
15 immunoprecipitate in suspension, then terminated and analysed as
16 described (Alessi et al., 1995). One unit of activity was that
17 amount of enzyme which catalysed the phosphorylation of 1 nmol of
18 Crosstide in 1 min. The phosphorylation of other peptides, histone
19 H2B and MBP were carried out in an identical manner. All the
20 Crosstide activity in HA-PKB α immunoprecipitates is catalysed by
21 PKB α (see Results) and the PKB α concentration in the
22 immunoprecipitates was estimated by densitometric scanning of
23 Coomassie blue-stained polyacrylamide gels, using bovine serum
24 albumin as a standard. Protein concentrations were determined by
25 the method of Bradford using bovine serum albumin as standard
26 (Bradford et al., 1976). Michaelis constants (K_m) and V_{max} values
27 were determined from double reciprocal plots of 1/V against 1/S,
28 where V is the initial rate of phosphorylation, and S is the
29 substrate concentration. The standard errors for all reported
30 kinetic constants were within $\leq \pm 20\%$, and the data is reported as
31 mean values for 3 independent determinations. Fig 16 shows the
32 results relative to those obtained for unstimulated PKB α .

33
34 **Tryptic digestion of histone 2B phosphorylated by PKB α .** Histone
35 H2B (30 μ M) was phosphorylated with 0.2 U/ml HA-PKB α . After 60 min
36 0.2 vol of 100% (by mass) trichloroacetic acid was added, and the
37 sample incubated for 1 h on ice. The suspension was centrifuged
38 for 10 min at 13, 000 x g, the supernatant discarded and the
39 pellet washed five times with 0.2 ml of ice cold acetone. The
40 pellet was resuspended in 0.3 ml of 50 mM Tris/HCl pH 8.0, 0.1%
41 (by vol) reduced Triton-X100 containing 2 μ g of alkylated trypsin
42 and, after incubation for 16 h at 30 °C, the digest was centrifuged
43 for 5 min at 13, 000 x g. The supernatant, containing 95% of the

1 ³²P-radioactivity, was chromatographed on a Vydac C18 column
2 equilibrated with 0.1% (by vol) trifluoroacetic acid (TFA) in
3 water. With reference to the results shown in Fig 17, the columns
4 were developed with a linear acetonitrile gradient (diagonal line)
5 at a flow rate of 0.8 ml / min and fractions of 0.4 ml were
6 collected. (A) Tryptic peptide map of ³²P-labelled histone H2B,
7 70% of the radioactivity applied to the column was recovered from
8 the major ³²P-peptide eluting at 19.5% acetonitrile. (B) A portion
9 of the major ³²P-peptide (50 pmol) was analysed on an Applied
10 Biosystems 476A sequencer, and the phenylthiohydantoin (Pth) amino
11 acids identified after each cycle of Edman degradation are shown
12 using the single letter code for amino acids. A portion of the
13 major ³²P-peptide (1000 cpm) was then coupled covalently to a
14 Sequelon arylamine membrane and analysed on an Applied Biosystems
15 470A sequencer using the modified programme described in (Stokoe
16 et al., 1992). ³²P radioactivity was measured after each cycle of
17 Edman degradation.
18
19

Table 7.1

Molecular basis for the substrate specificity of PKB α

The phosphorylated residue is shown in boldface type, the altered residue is underlined. V(100 μ M) is the relative rate of phosphorylation at 0.1 mM peptide relative to peptide 1. ND, not determined. *An alanine residue was added to the C-terminal of the peptide RPRTSSP, since we have experienced difficulty in synthesizing peptides terminating in proline.

A	Peptides	K _m (μ M)	V _{max} (U/mg)	V(0.1 mM)
1.	GRPRTSSFAEG	4	250	100
2.	RPRTSSFA	8	305	109
3.	GRPRTSSF	8	385	129
4.	RPRTSSF	5	260	105
5.	RPRTSTF	30	243	78
6.	RPRTSAF	-	0	
7.	PRTSSF	-	0	
8.	RPRTSS	>500	ND	2
9.	KPRTSSF	>500	ND	4
10.	RPKTSSF	>500	ND	2
B				
1.	RPRTSSF	5	260	105
2.	RPRTSSL	8	278	104
3.	RPRTSSV	21	300	102
4.	RPRTSSA	250	265	30
5.	RPRTSSK	80	308	67
6.	RPRTSSE	>500	ND	9
7.	RPRTSSPA*	-	0	
C				
1.	RPRTSSF	5	260	105
2.	RPRASSF	12	230	89
3.	RPRVSSF	25	273	77
4.	RPRGSSF	60	163	37
5.	RPRNSSF	>500	ND	21
6.	RPRTASF	20	213	83
7.	RPRTGSF	25	233	77
8.	RPRTVSF	30	365	89
9.	RPRTNSF	30	300	81
10.	RPRAASF	25	215	77
11.	RPRGGSF	105	345	55
12.	RPRGASF	105	160	37
13.	RPRAGSF	49	114	70

Table 7.2 Comparison of the substrate specificities of PKB α , MAPKAP kinase-1, and p70S6 kinase.
 Peptides 1 and 2 are very good substrates for MAPKAP kinase-1 and p70 S6 kinase, and peptide 3 is a relatively specific substrate for p70 S6 kinase [16]. *Data reported previously [16]; ND, not determined.

A	Peptide	Protein kinase B α		MAPKAP kinase-1		p70 S6 kinase	
		K _m (mM)	V _{max} (U/mg)	K _m (mM)	V _{max} (U/mg)	K _m (mM)	V _{max} (U/mg)
1.	KKKNR T LSVA	185	270	0.2*	1550*	3.3*	890*
2.	KKNR T LSVA	80	300	0.7*	1800*	1.5*	1520*
3.	KKRNK T LSVA	>500	ND	17*	840*	34*	760*
4.	KKNR T LT V	388	330	40*	270*	4.8*	1470*

B	Peptide	K _m (mM)	V _{max} (U/mg)	K _m (mM)	V _{max} (U/mg)	K _m (mM)	V _{max} (U/mg)
1.	GRPRTSSFAEG	4	250	2	790	3	1270
2.	RPRTSSF	5	260	12	840	125	705
3.	RPRTSTF	30	240	>500	ND	211	590
4.	RPRAASF	25	215	20	1020	>500	ND
5.	RPRAATF	25	230	>500	ND	>500	ND

1 The following documents are incorporated herein by reference.

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Claims:

1 The use of a composition of PKB, its analogues, isoforms, inhibitors, activators and/or the functional equivalents thereof, to regulate glycogen metabolism and/or protein synthesis.

2 The use of a composition of PKB, its analogues, isoforms, inhibitors, activators and/or the functional equivalents thereof, for the manufacture of a medicament to regulate glycogen metabolism and/or protein synthesis.

3 The use as claimed in claim 1 or claim 2, to combat disease states where glycogen metabolism and/or protein synthesis exhibits abnormality.

4 The use as claimed in claim 1, 2 or 3, to combat diabetes.

5 The use as claimed in any preceding claim, to combat cancer.

6 The use as claimed in claim 5, wherein the cancer is breast, pancreatic or ovarian cancer.

7 The use as claimed in any preceding claim, wherein the PKB is PKB α , β or γ , an analogue, isoform, inhibitor, activator or a functional equivalent thereof.

8 The use as claimed in any preceding claim, wherein the PKB, its analogue, isoform, or functional equivalent is modified at one or both of amino acids 308 and 473 by phosphorylation and/or mutation.

1 9 A composition of PKB, its analogues, isoforms,
2 inhibitors, activators and/or the functional
3 equivalents thereof.

4

5 10 A peptide having or including the amino acid
6 sequence Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is
7 any amino acid, Yaa and Zaa are any amino acid, and Hyd
8 is a large hydrophobic residue, or a functional
9 equivalent of such a peptide.

10

11 11 A peptide as claimed in claim 10, wherein Hyd is
12 Phe or Leu, or a functional equivalent thereof.

13

14 12 A peptide as claimed in claim 10 or claim 11,
15 wherein Yaa or Zaa or both are an amino acid other than
16 glycine.

17

18 13 A peptide as claimed in claim 10, having the amino
19 acid sequence GRPRTSSFAEG, or a functional equivalent
20 thereof.

21

22 14 A method of identifying agents able to influence
23 the activity of GSK3, said method comprising:

24

25 a. exposing a test substance to a substrate of GSK3;
26 and

27 b. detecting whether said substrate has been
28 phosphorylated.

29

30 15 A method of identifying agents which influence the
31 activity of PKB, comprising:

32

33 a. exposing a test substance to a sample containing
34 PKB, to form a mixture;

35 b. exposing said mixture to a peptide as claimed in
36 claim 10, 11, 12 or 13; and

- 1 c. detecting whether (and, optionally, to what
2 extent) said peptide has been phosphorylated.
3
- 4 16 A method as claimed in claim 14 or 15, wherein the
5 extent of phosphorylation of the peptide is determined.
6
- 7 17 A method as claimed in claim 15, wherein the
8 phosphorylation state(s) of one or both of amino acids
9 308 and 473 on PKB is determined.
10
- 11 18 A method as claimed in any one of claims 14 to 17,
12 wherein the test substance is an analogue, isoform,
13 inhibitor, or activator of PKB.
14
- 15 19 A method as claimed in any one of claims 14 to 18,
16 wherein steps a or b (or both) are carried out in the
17 presence of divalent cations and ATP.
18
- 19 20 A method of treatment of the human or non-human
20 animal body, said method comprising administering PKB,
21 its analogues, inhibitors, stimulators or functional
22 equivalents thereof to said body.
23
- 24 21 A method as claimed in claim 20, to combat disease
25 states where glycogen metabolism and/or protein
26 synthesis exhibits abnormality.
27
- 28 22 A method as claimed in claim 20 or 21, to combat
29 diabetes.
30
- 31 23 A method as claimed in claim 20 or 22, to combat
32 cancer.
33
- 34 24 A method as claimed in claim 23, wherein the
35 cancer is breast, pancreatic or ovarian cancer.
36

1 25 A method as claimed in any one of claims 20 to 24,
2 wherein the PKB is PKB α , β or γ , an analogue, isoform,
3 inhibitor, activator or a functional equivalent
4 thereof.

5
6 26 An agent capable of influencing the activity of
7 PKB, its isoforms, analogues and/or functional
8 equivalents, by modifying amino acids 308 and/or 473 by
9 phosphorylation or mutation.

10
11 27 A method of determining the ability of a substance
12 to affect the activity or activation of PKB, the method
13 comprising exposing the substance to PKB and
14 phosphatidyl inositol polyphosphate and determining the
15 interaction between PKB and the phosphatidyl inositol
16 polyphosphate.

17
18 28 A method of determining the ability of a substance
19 to combat diabetes, cancer, or any disorder which
20 involves irregularity of protein synthesis or glycogen
21 metabolism, the method comprising exposing the
22 substance to PKB and phosphatidyl inositol
23 polyphosphate and determining the interaction between
24 PKB and the phosphatidyl inositol polyphosphate.

25
26 29 A method as claimed in claim 27 or claim 28,
27 wherein the interaction between PKB and the
28 phosphatidyl inositol polyphosphate is measured by
29 assessing the phosphorylation state of PKB.

30
31 30 A method as claimed in claim 29, wherein the
32 phosphorylation state of PKB at T308 and/or S473 is
33 assessed.

34
35 31 A method of identifying activators or inhibitors
36 of GSK3 comprising exposing the substance to be tested

1 to GSK3 and determining the state of activation of
2 GSK3.

3

4 32 A method as claimed in claim 31 wherein the state
5 of activation of GSK3 is determined by assessing its
6 phosphorylation.

7

8 33 A method of determining the suitability of a test
9 substance for use in combatting diabetes, cancer, or
10 any disorder which involves irregularity of protein
11 synthesis or glycogen metabolism, the method comprising
12 exposing the substance to be tested to GSK3 and
13 determining the state of activation of GSK3.

14

15 34 A method for screening for inhibitors or
16 activators of enzymes that catalyse the phosphorylation
17 of PKB, the method comprising exposing the substance to
18 be tested to

19

- one or more enzymes upstream of PKB;

20

- PKB; and (optionally)

21

- nucleoside triphosphate

22

and determining whether (and optionally to what extent)

23

the PKB has been phosphorylated on T308 and/or S473.

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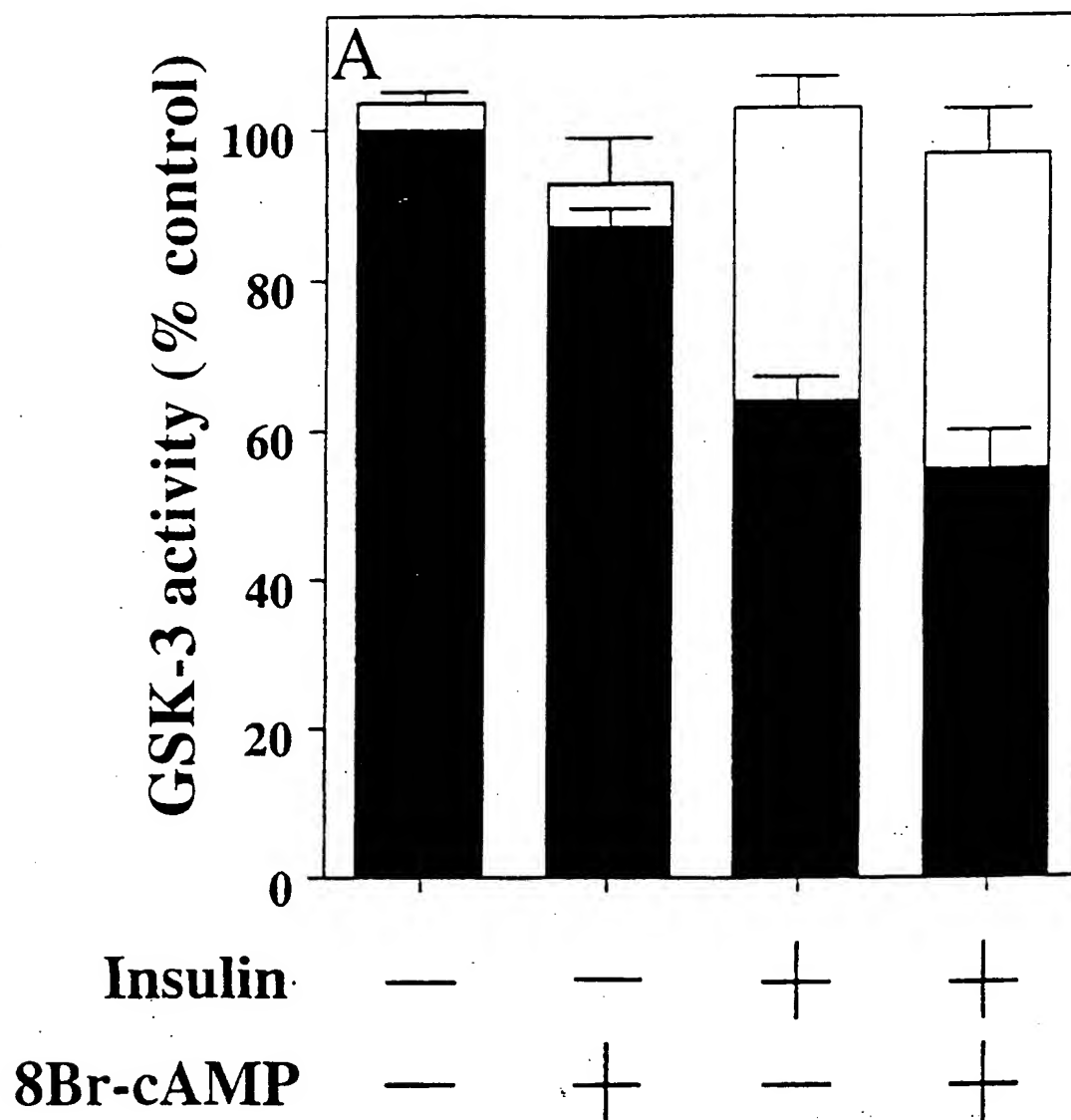
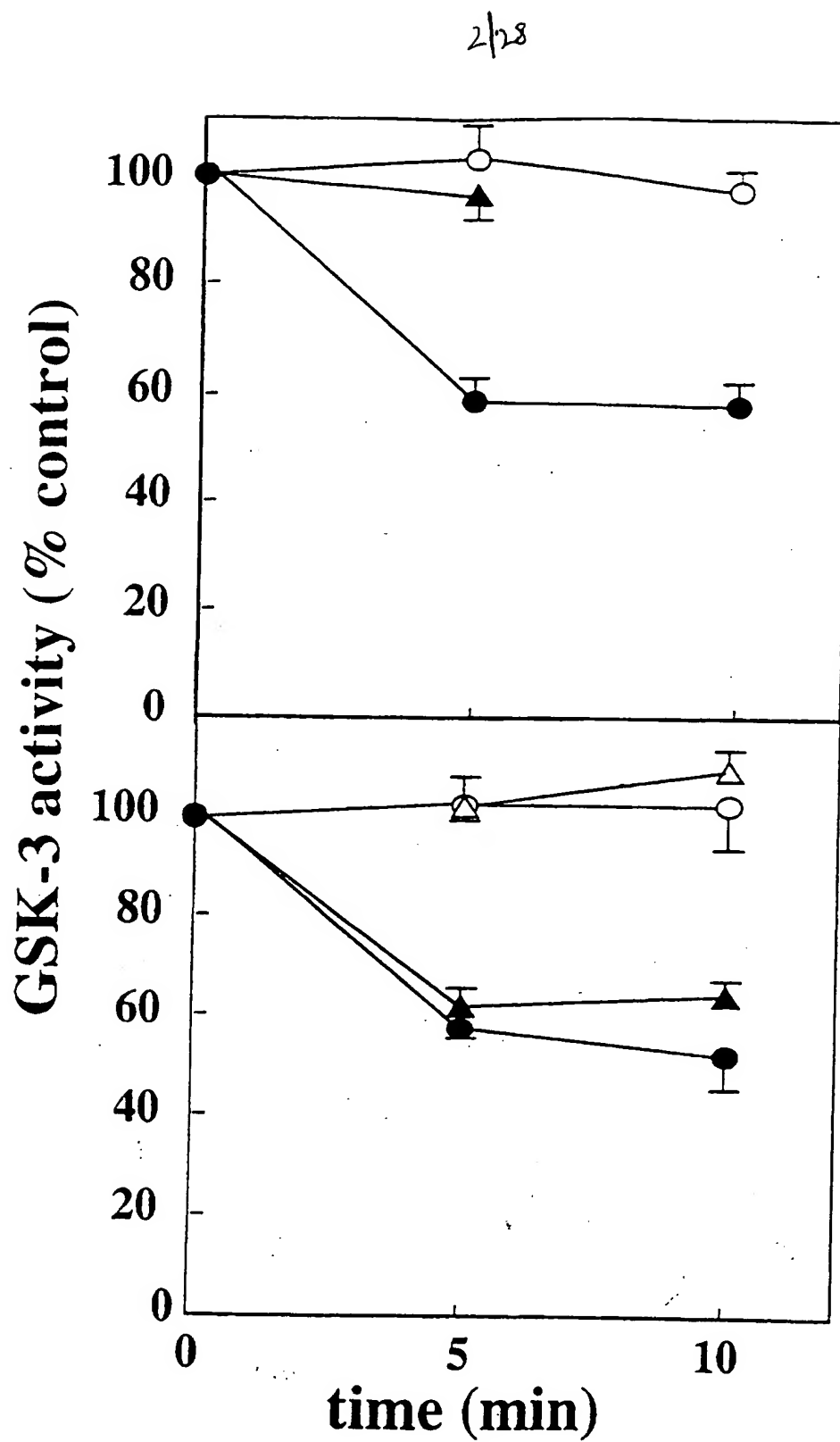


Fig. 1a



Figs. 1b & 1c

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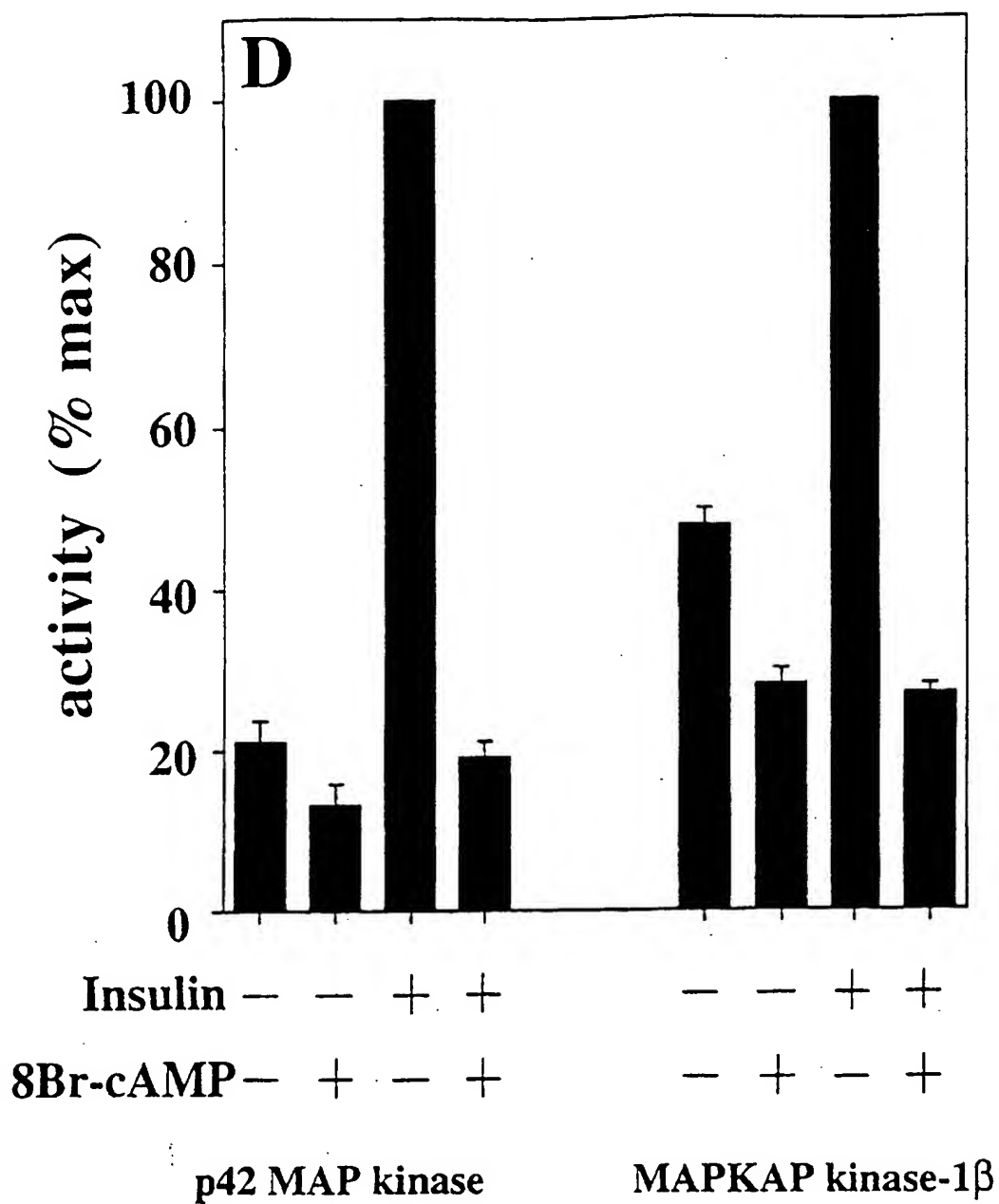


Fig. 1d

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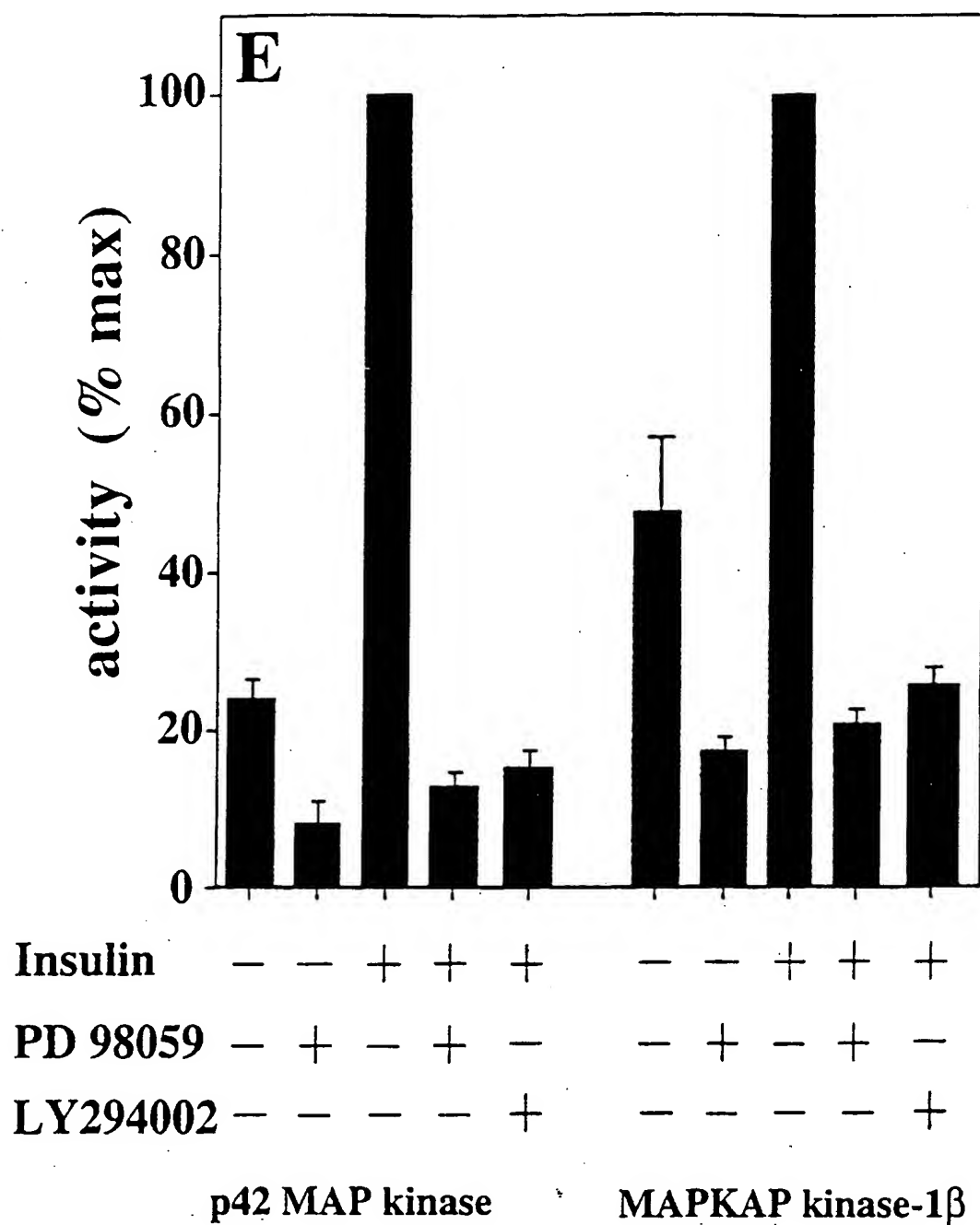


Fig. 1e

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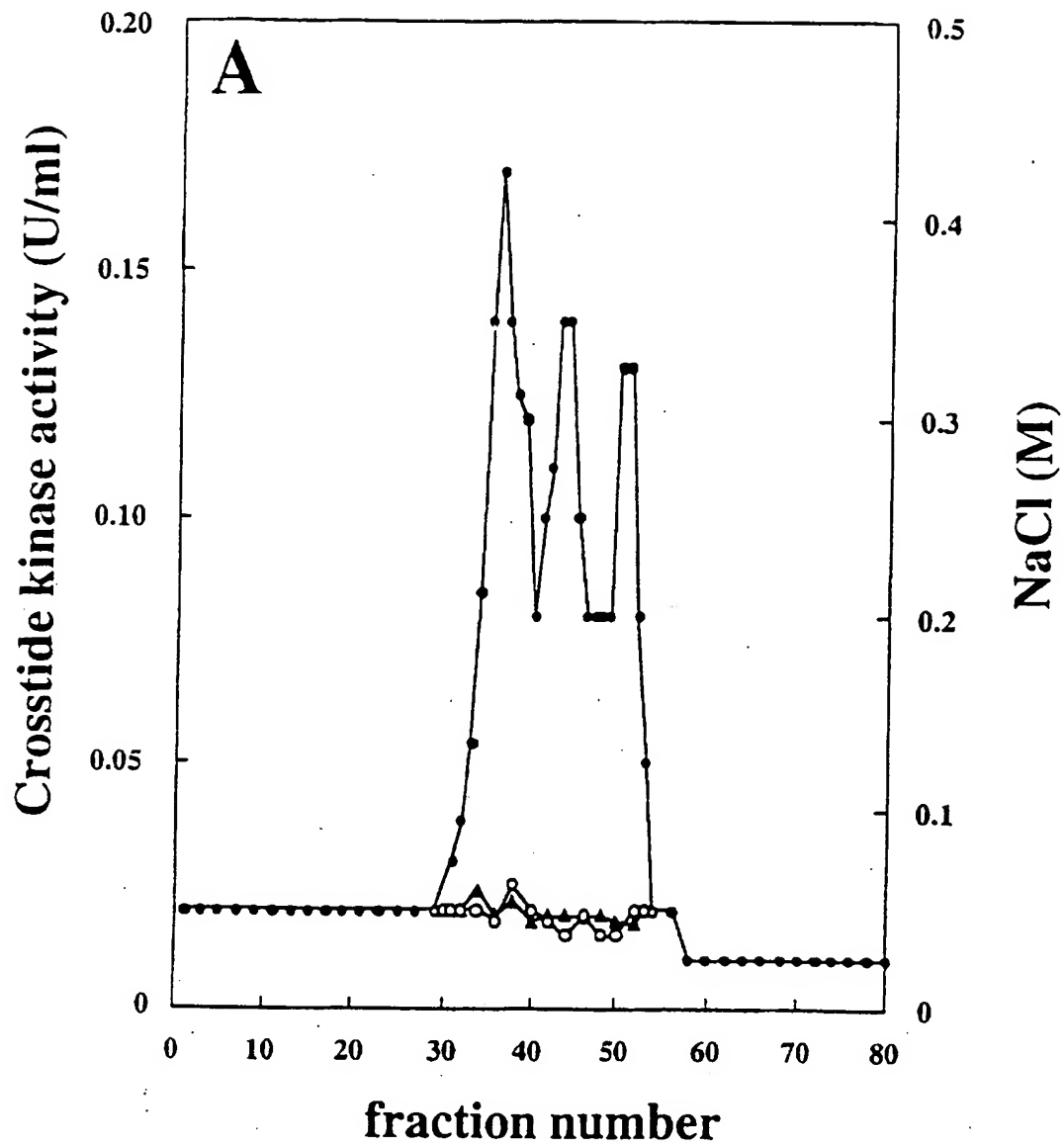
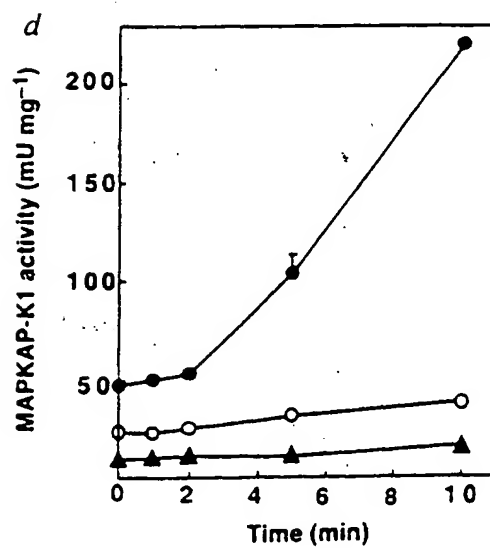
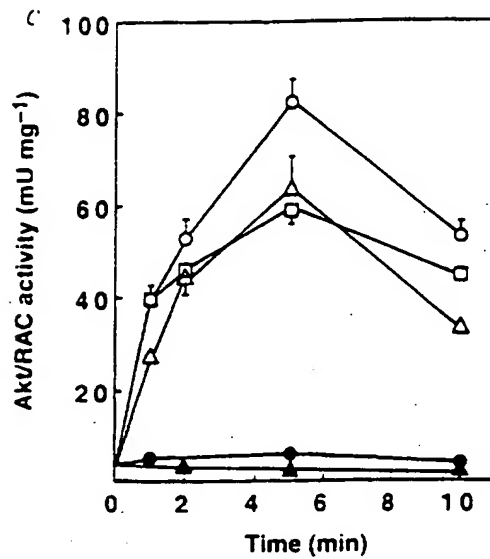


Fig. 2a

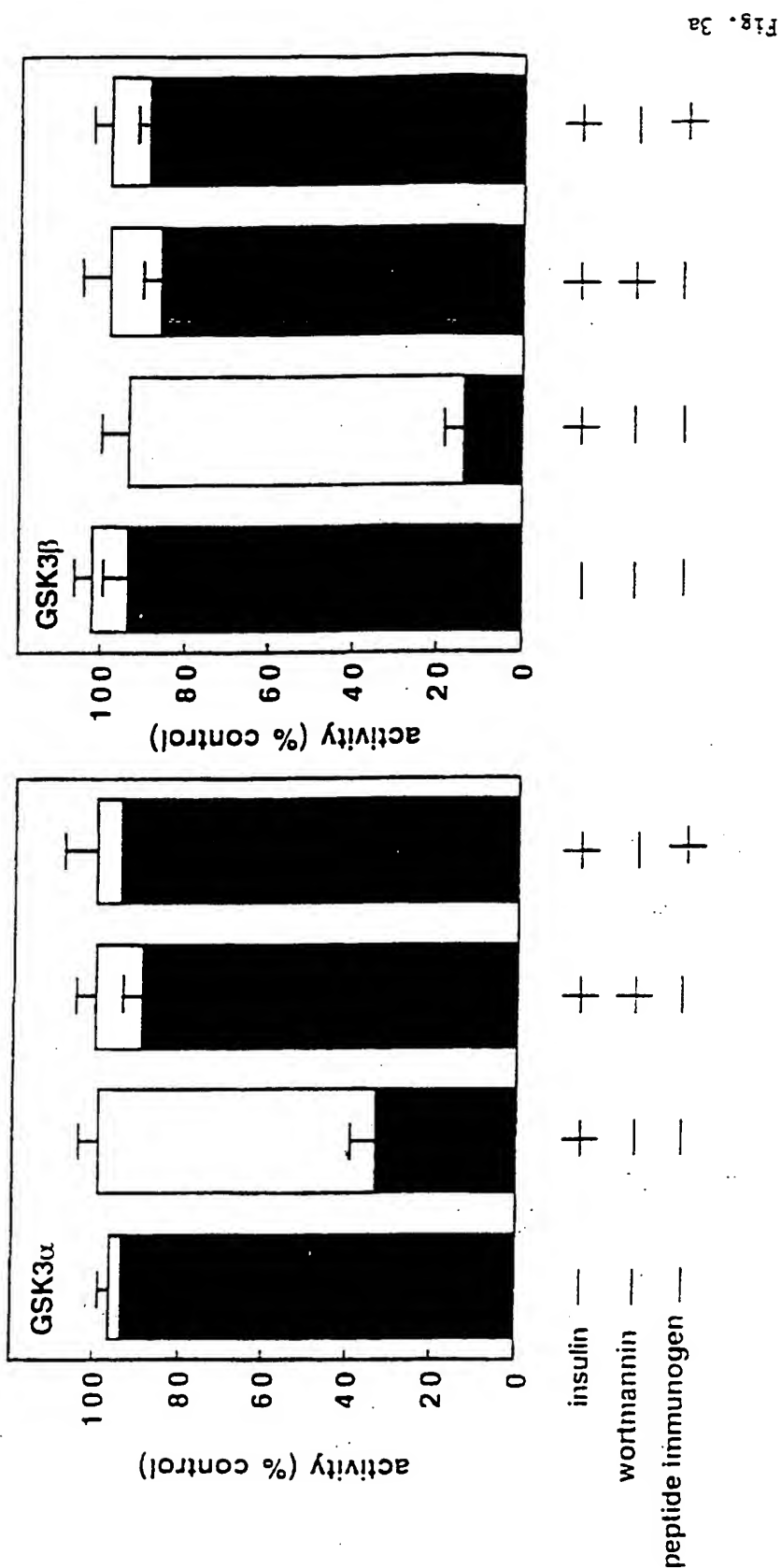
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b
 $M_r(K)$ 1 2 3 4 5 6
205—
97—
66—
43—



Figs. 2b, 2c & 2d

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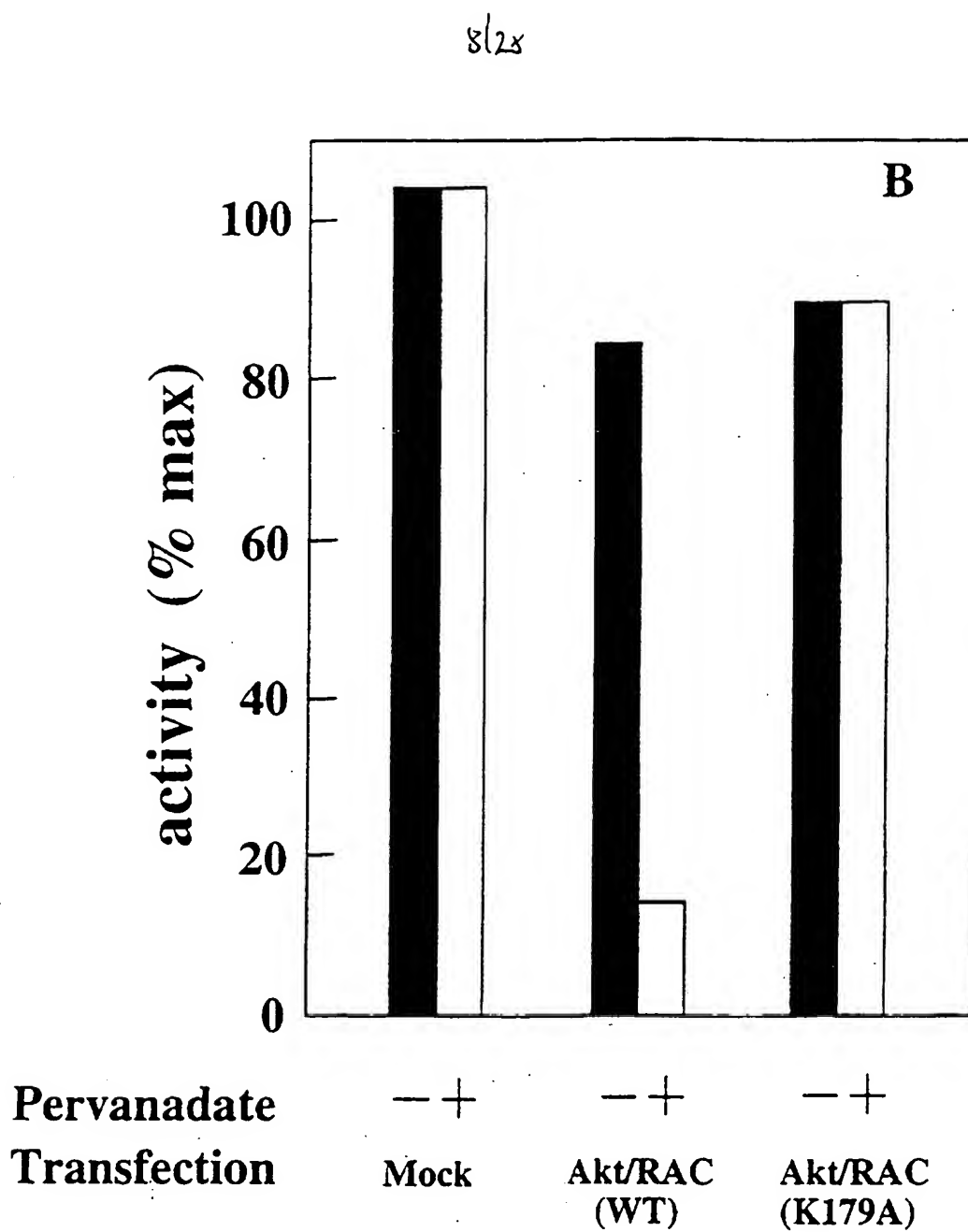
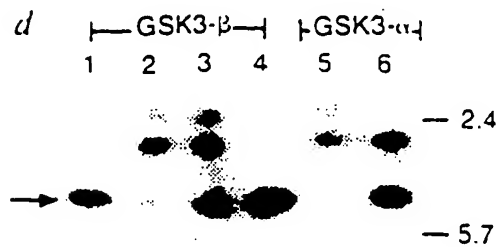
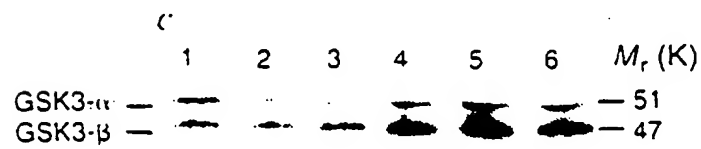
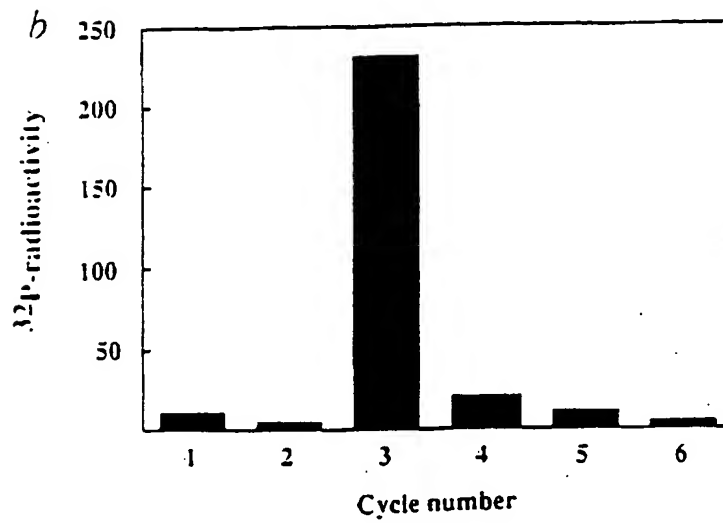
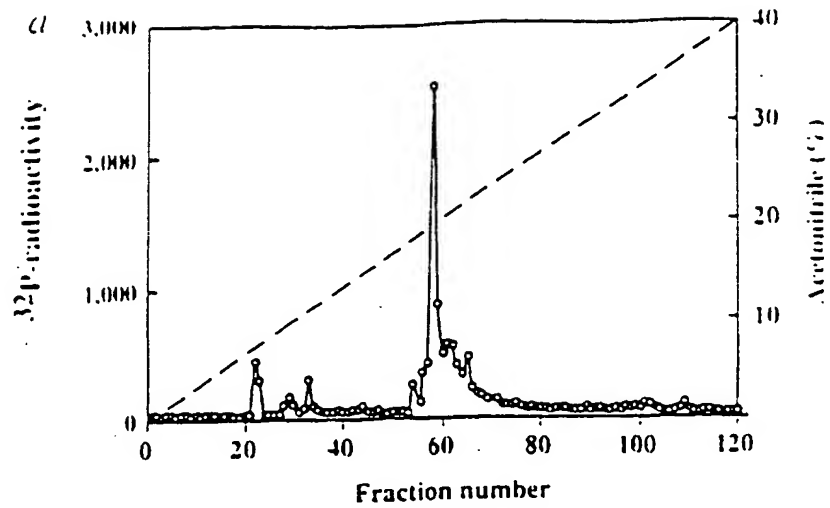


Fig. 3b

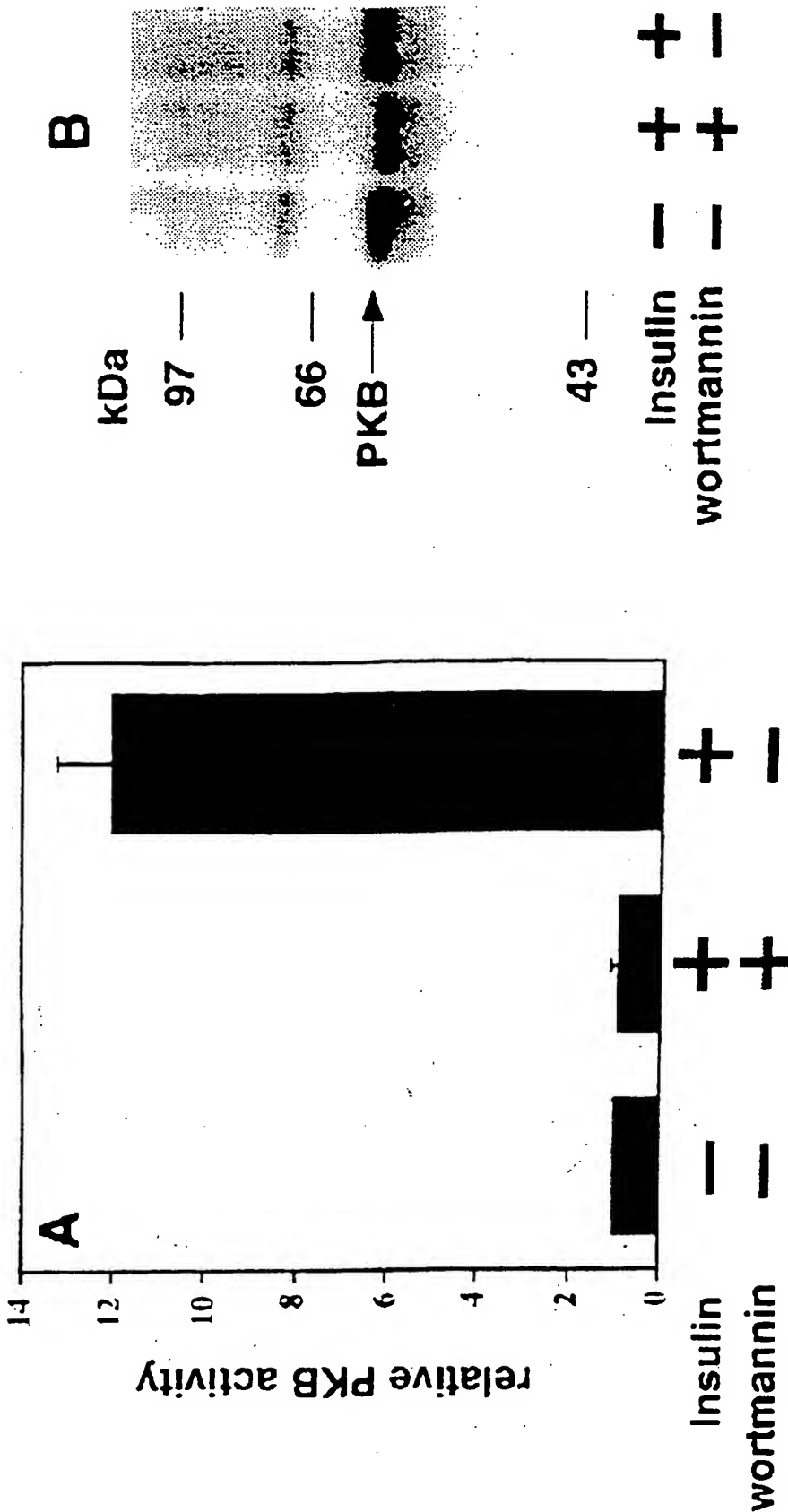
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Figs. 4a, 4b, 4c & 4d

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Fig. 5



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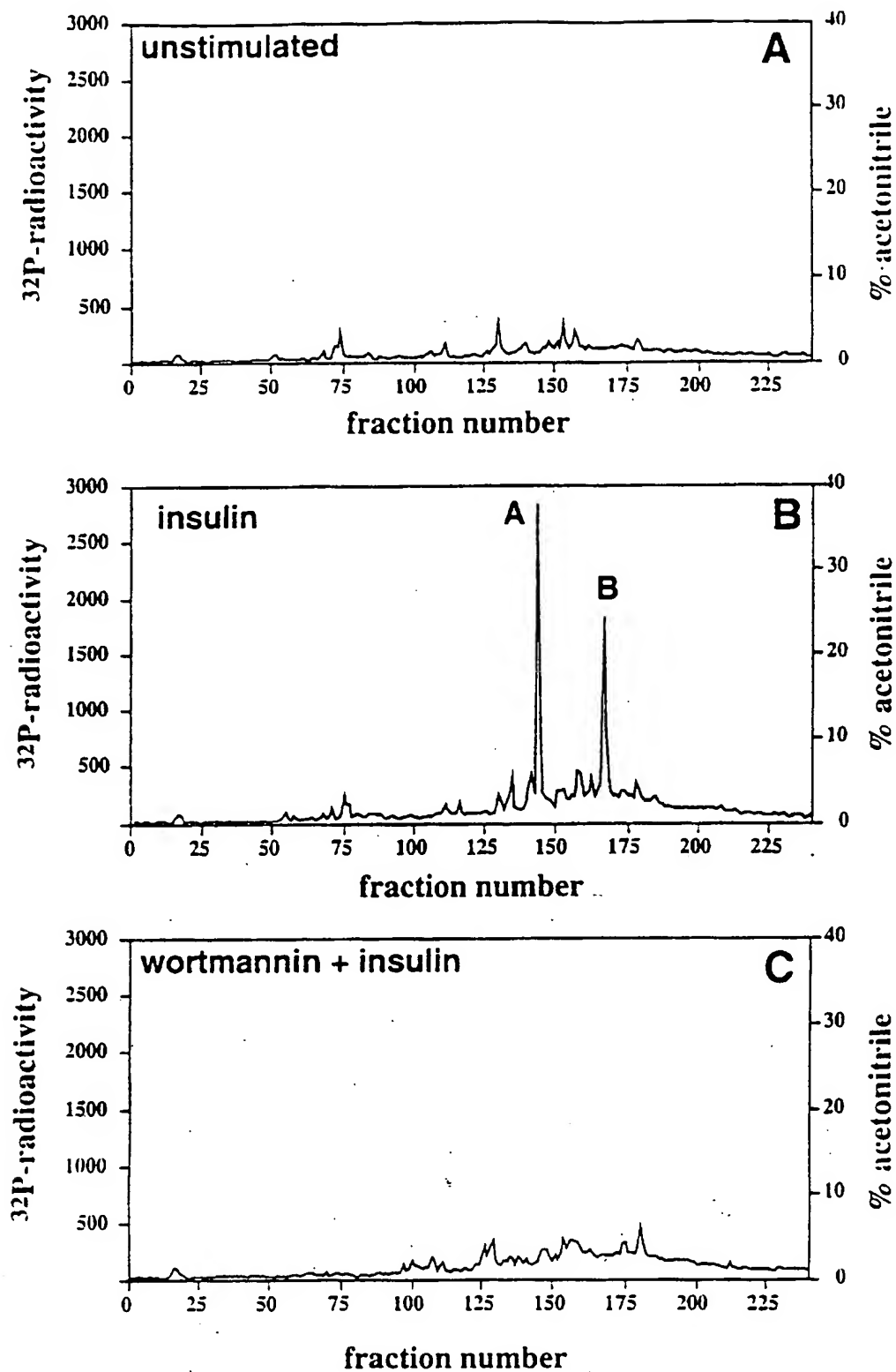


Fig. 6

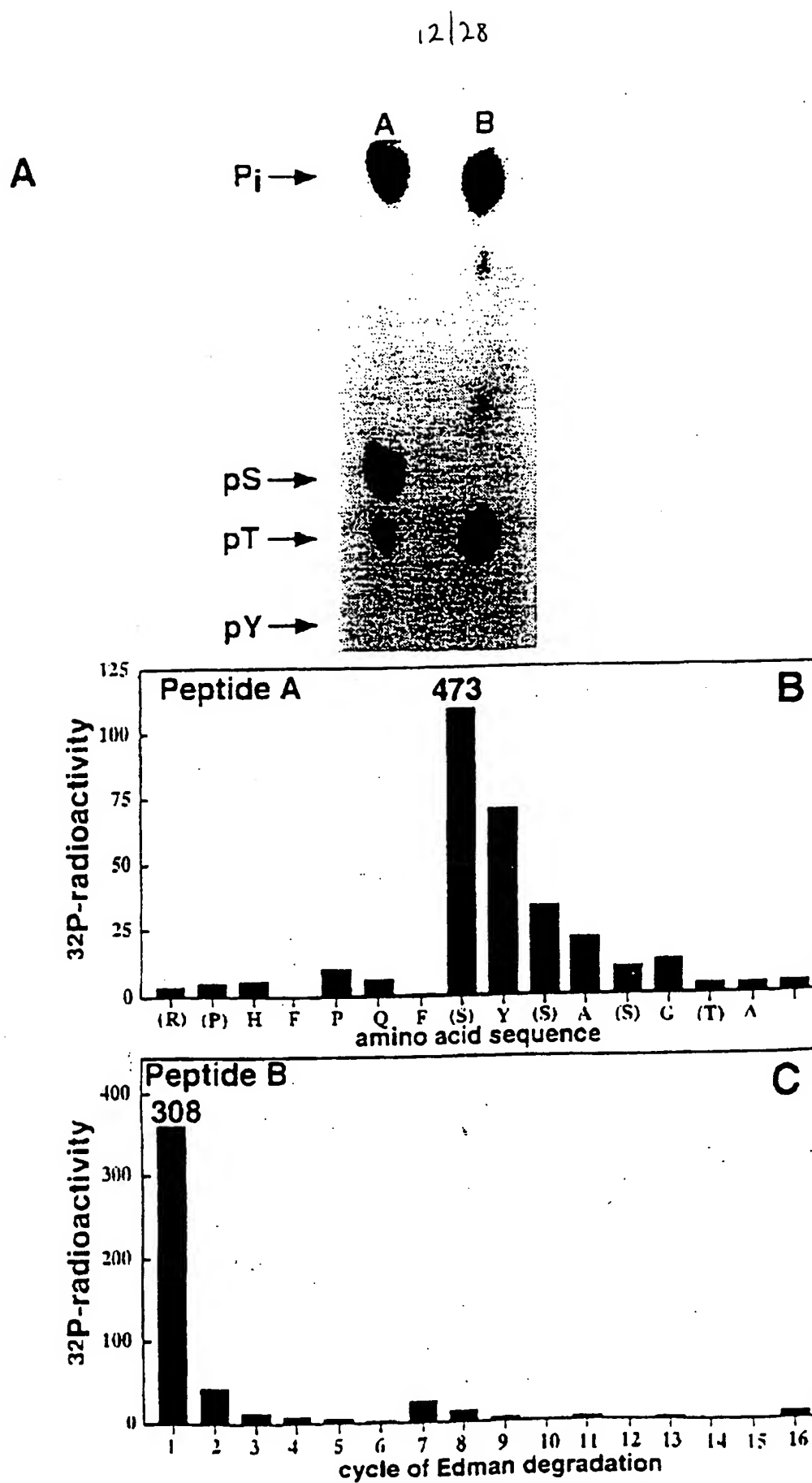
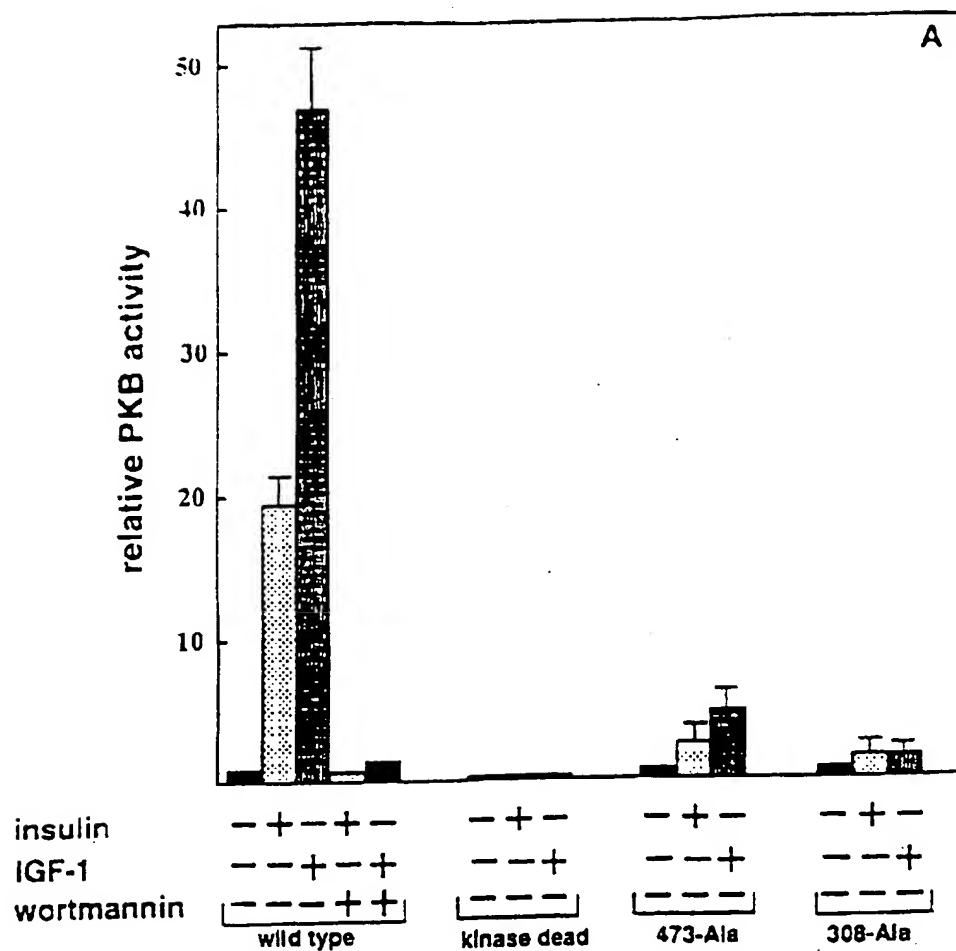


Fig. 7

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B

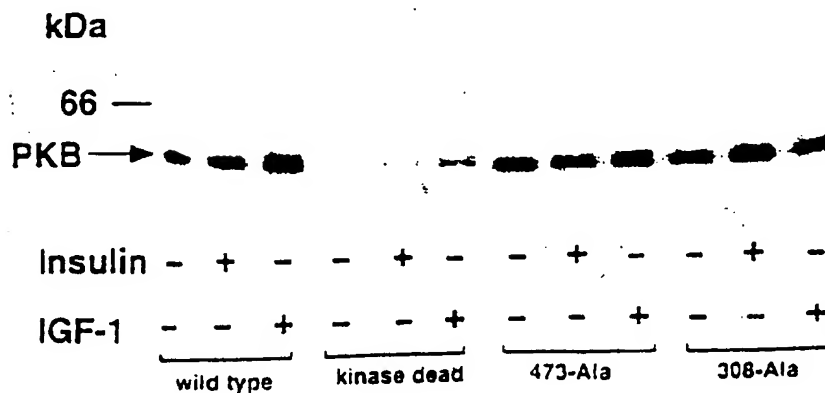


Fig. 8

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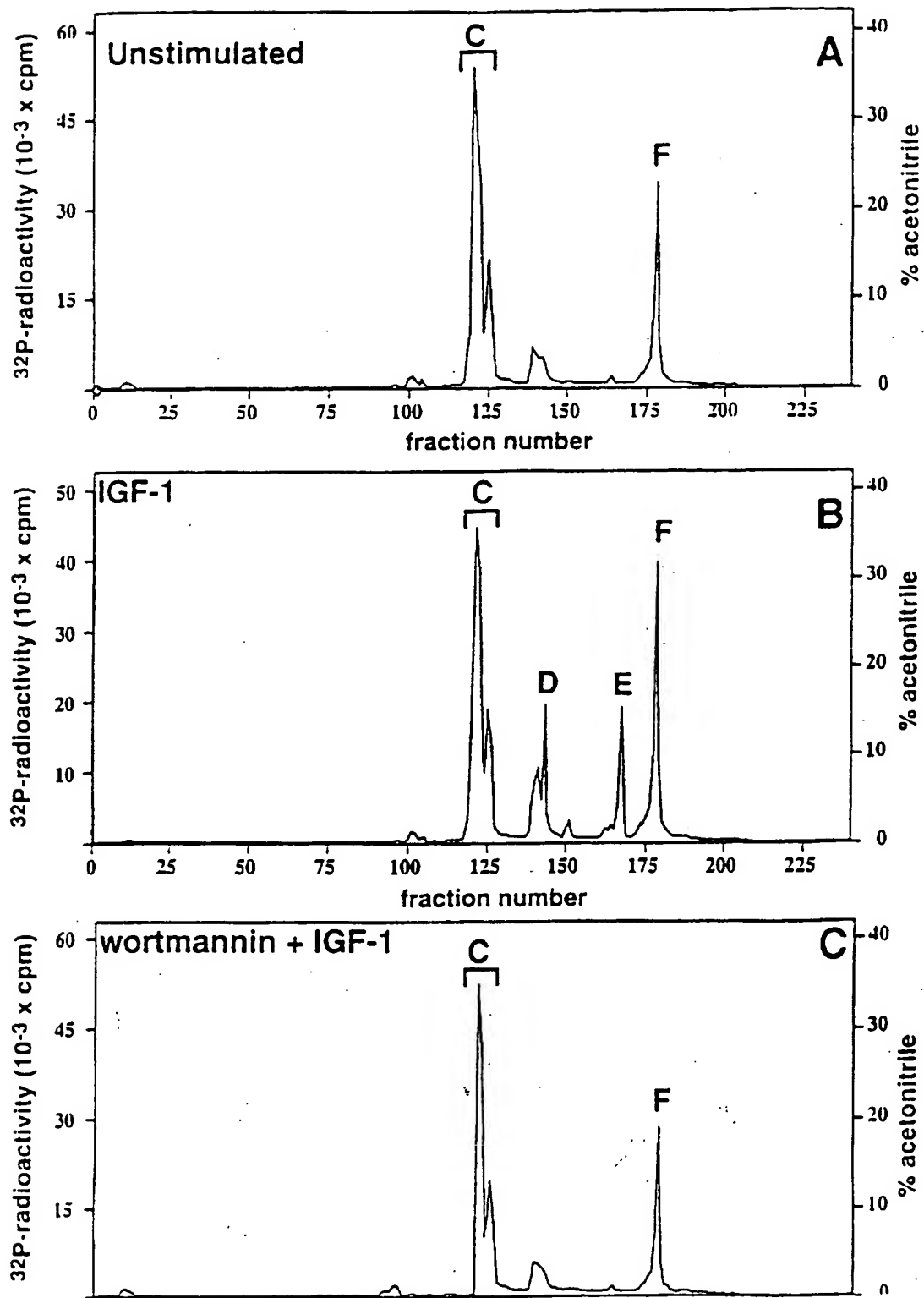
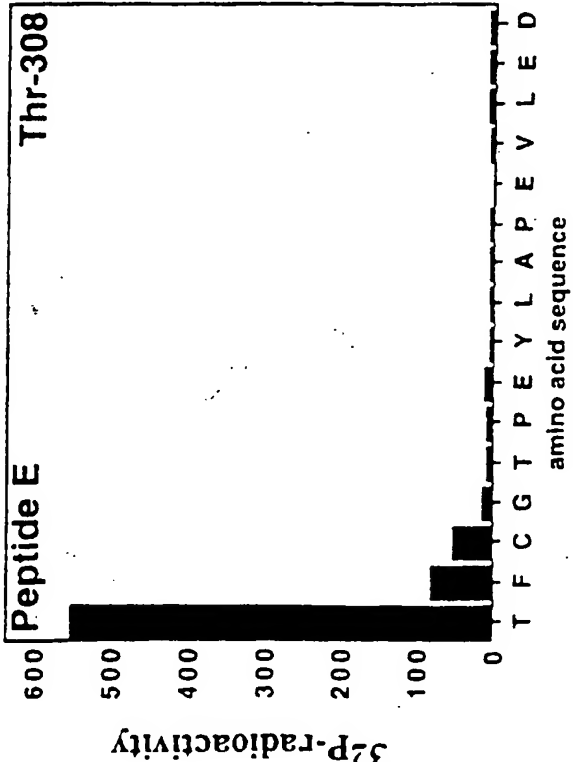
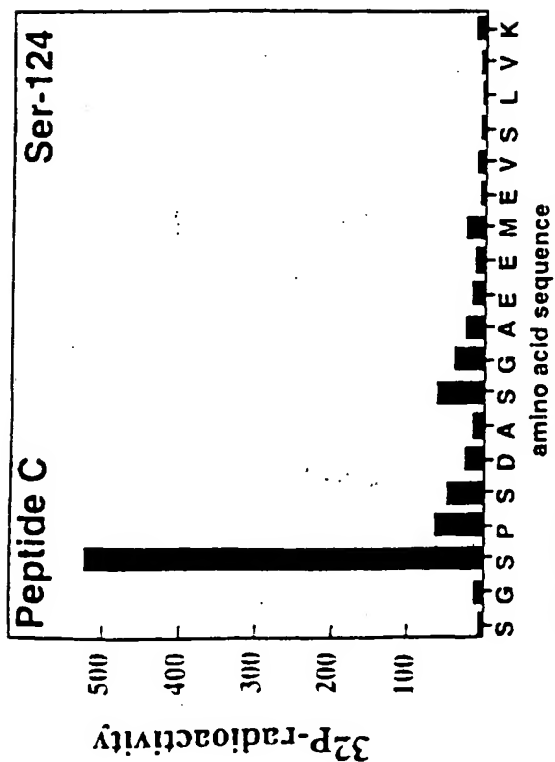
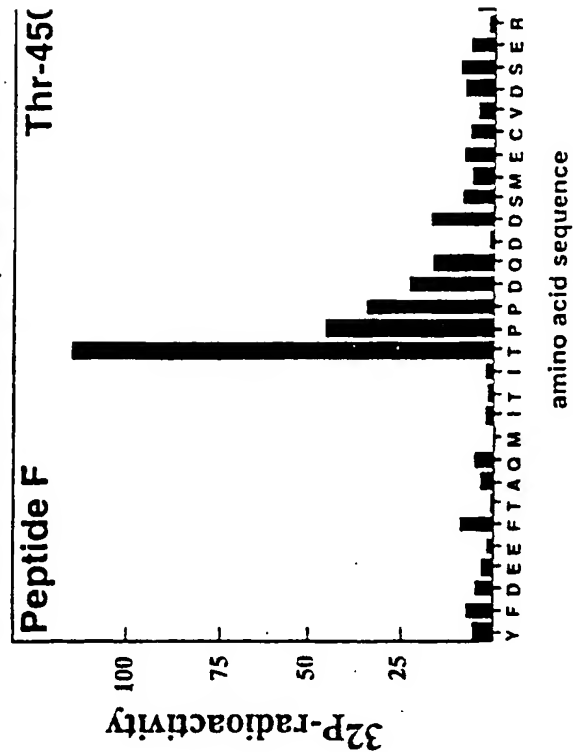
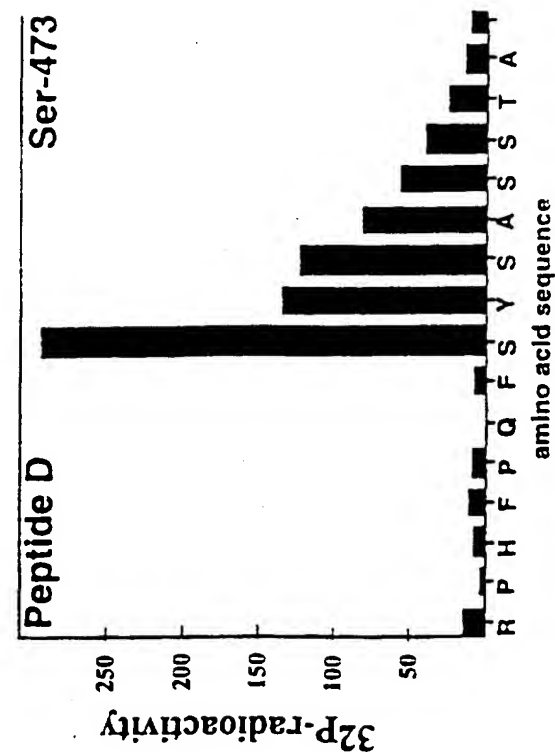


Fig. 9

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Fig. 10



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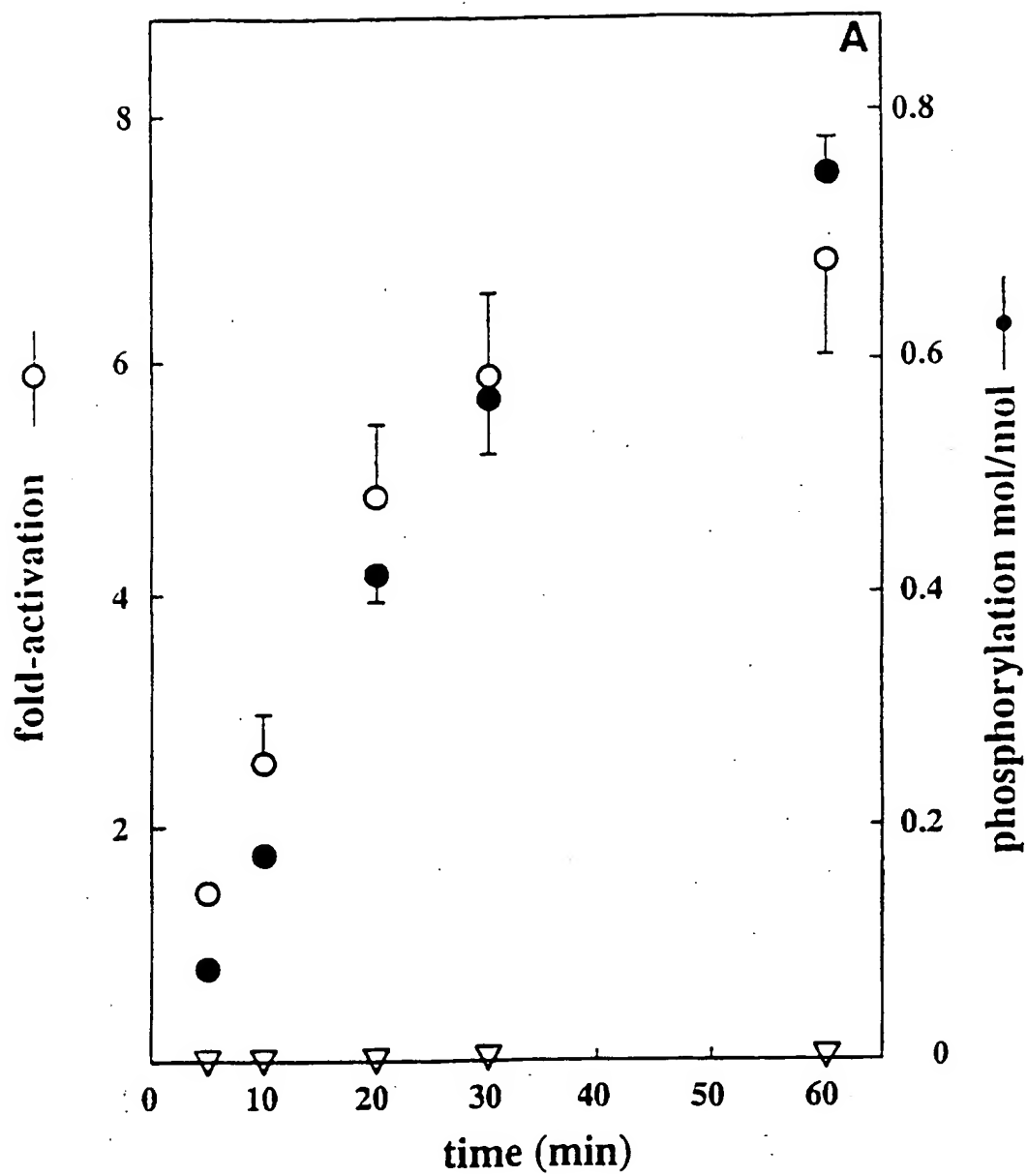
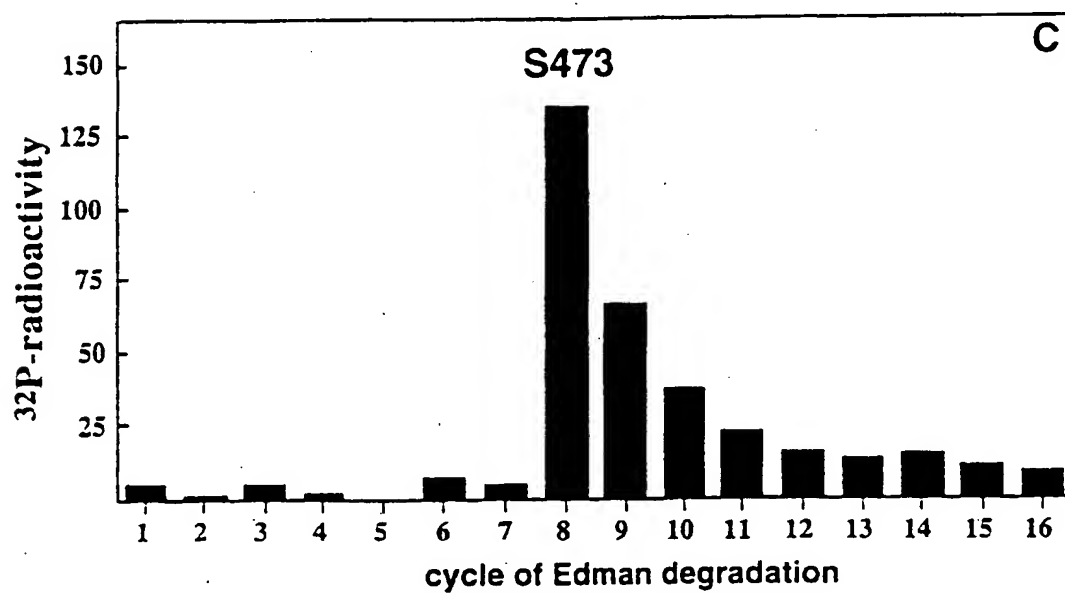
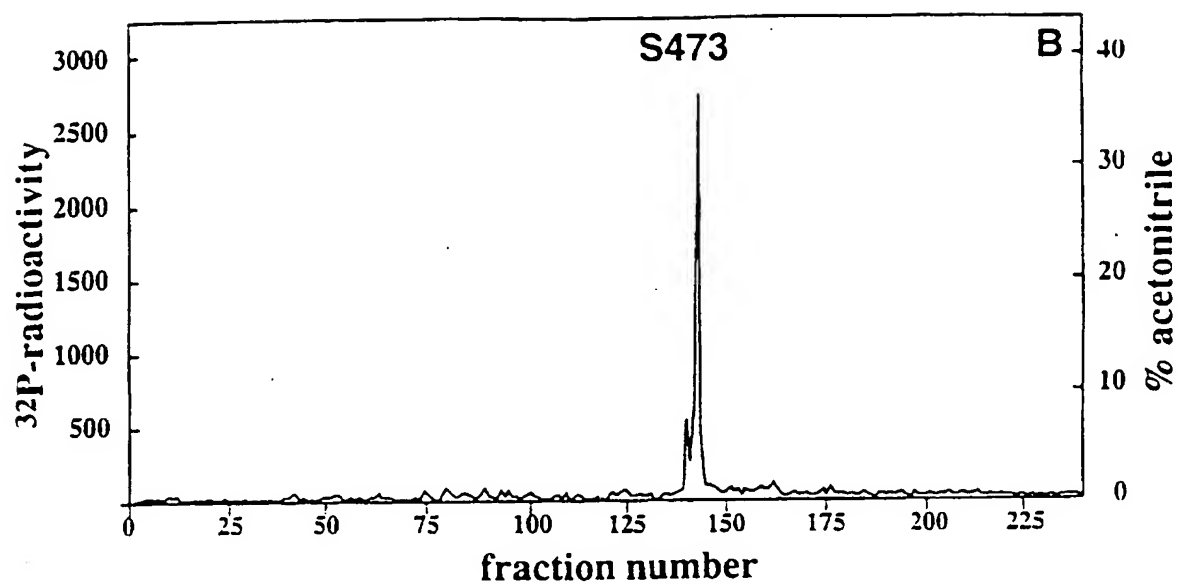


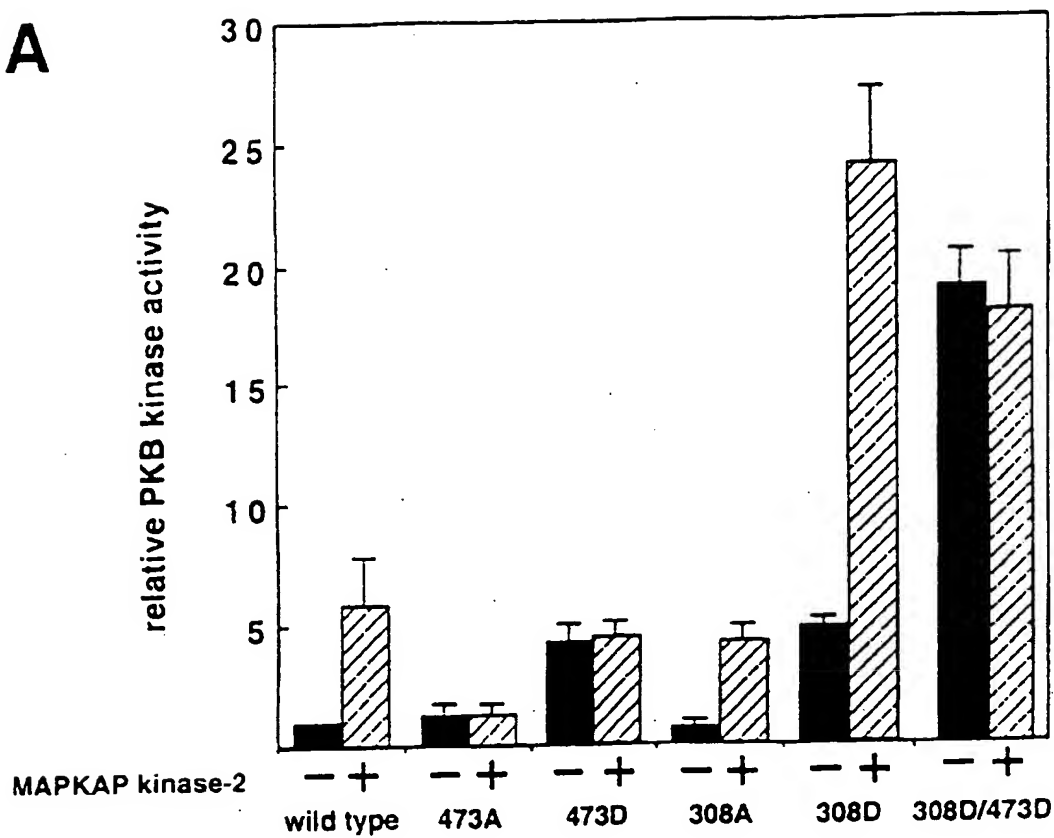
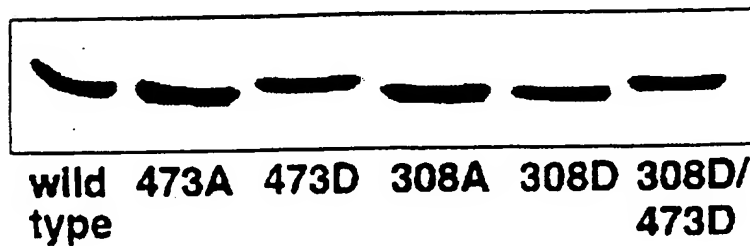
Fig. 11a

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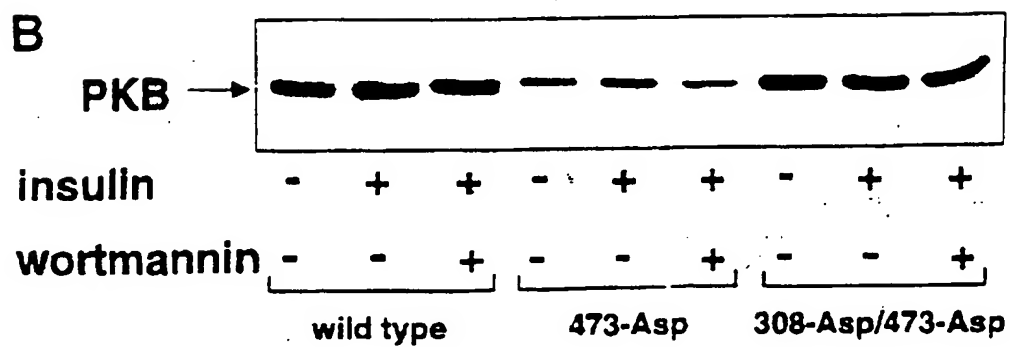
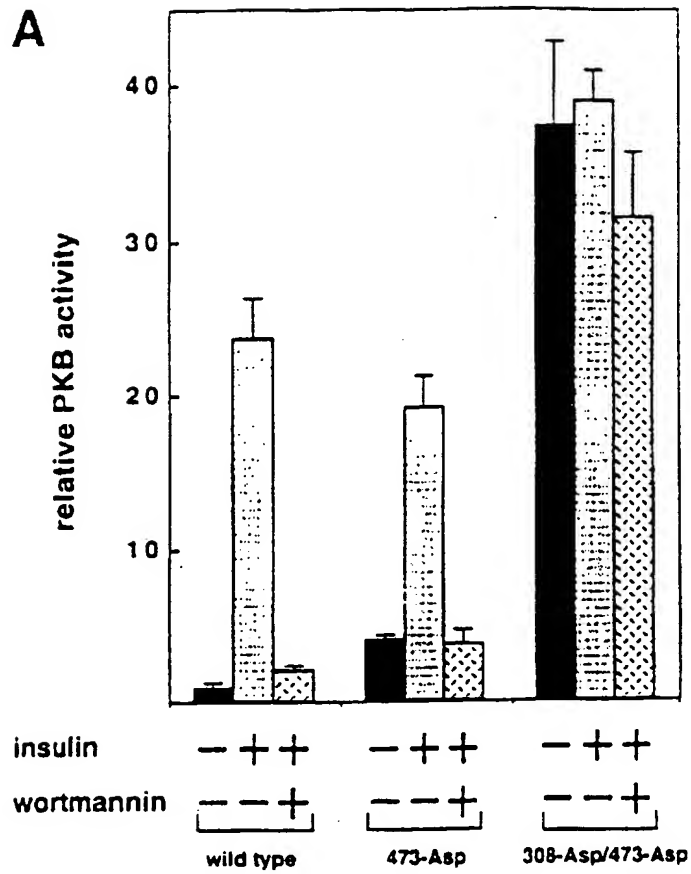
Figs. 11b & 11c

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A**B****PKB** →

Figs. 12a & 12b

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Figs. 13a & 13b

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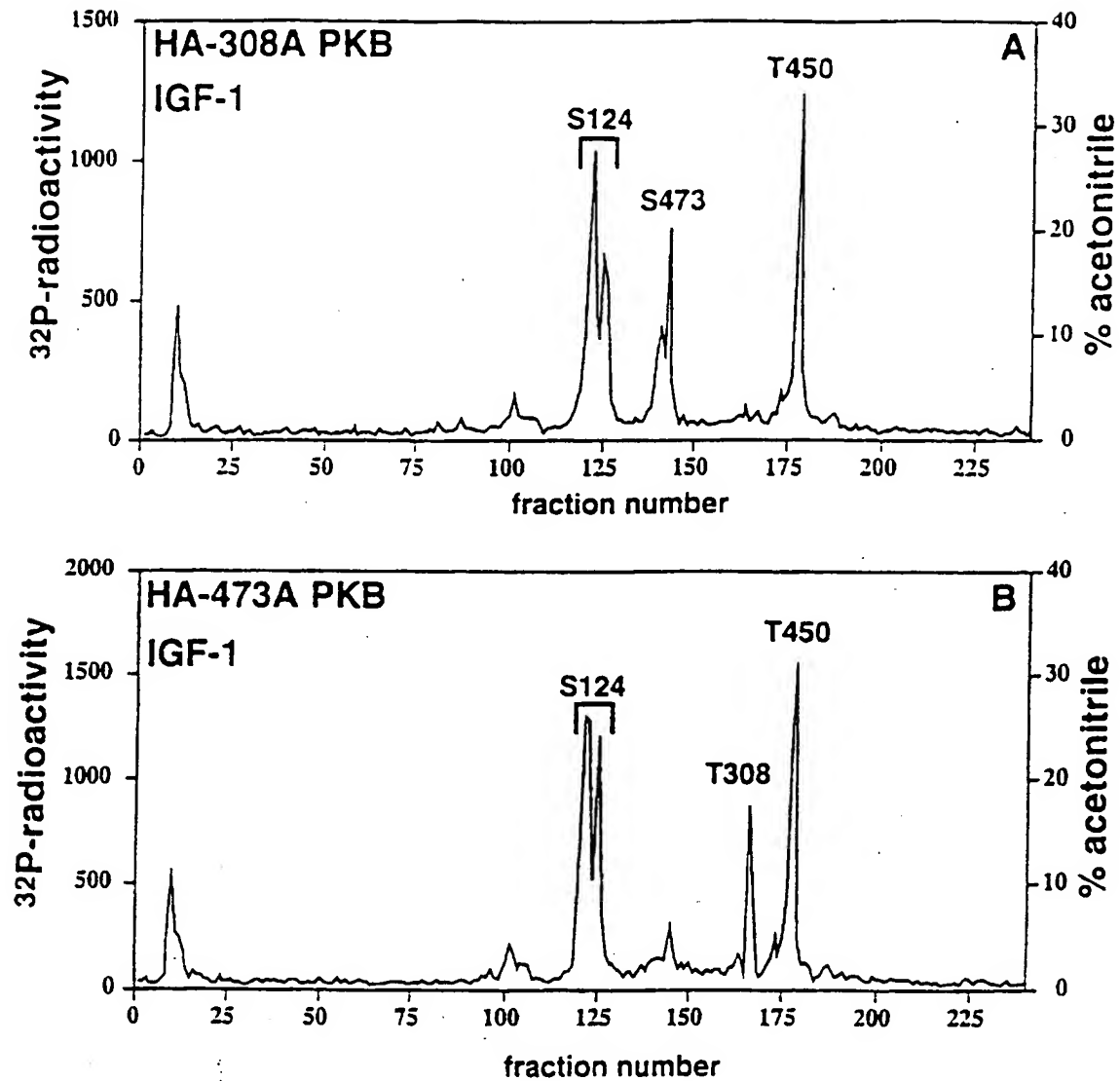


Fig. 14

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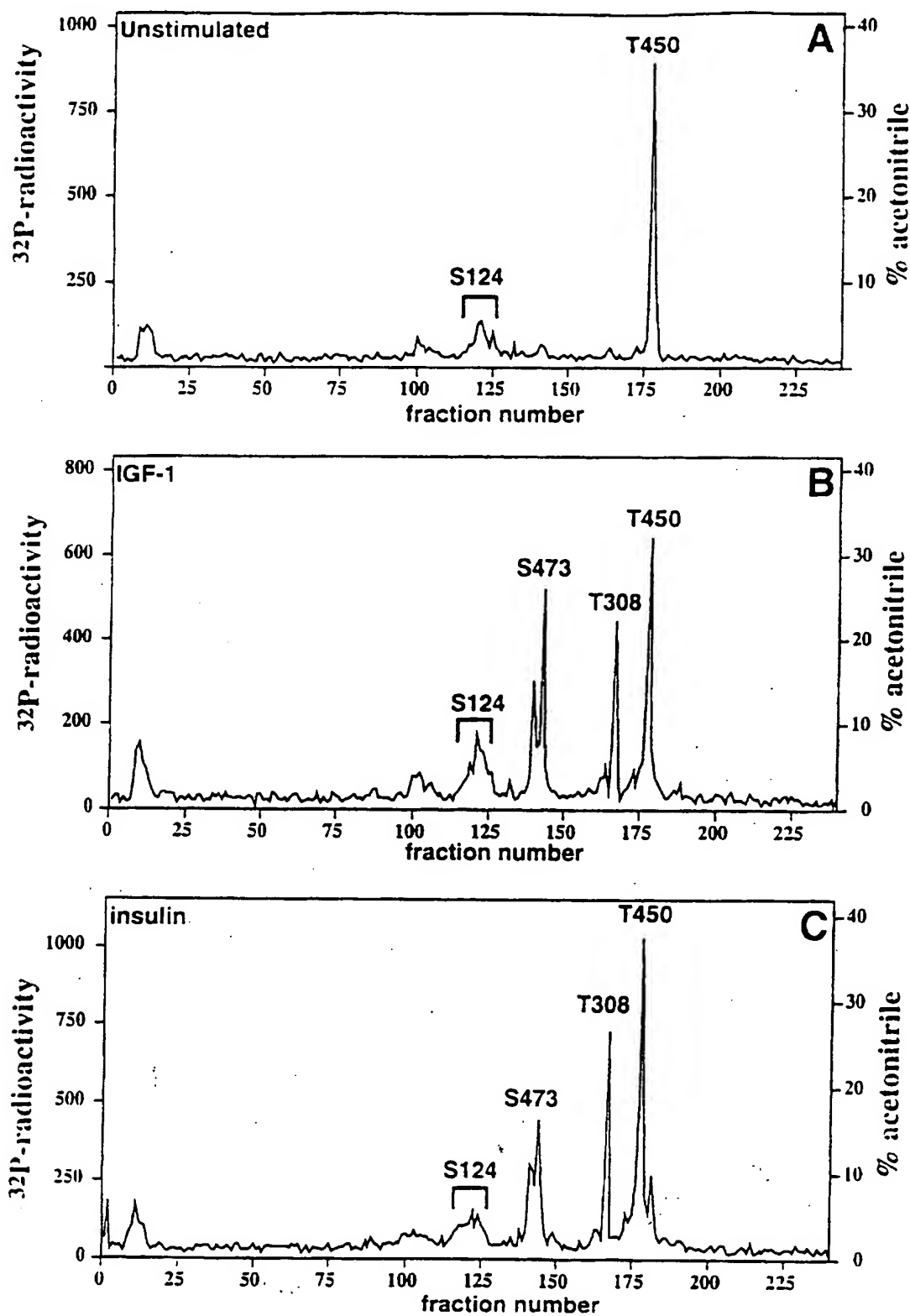


Fig. 15

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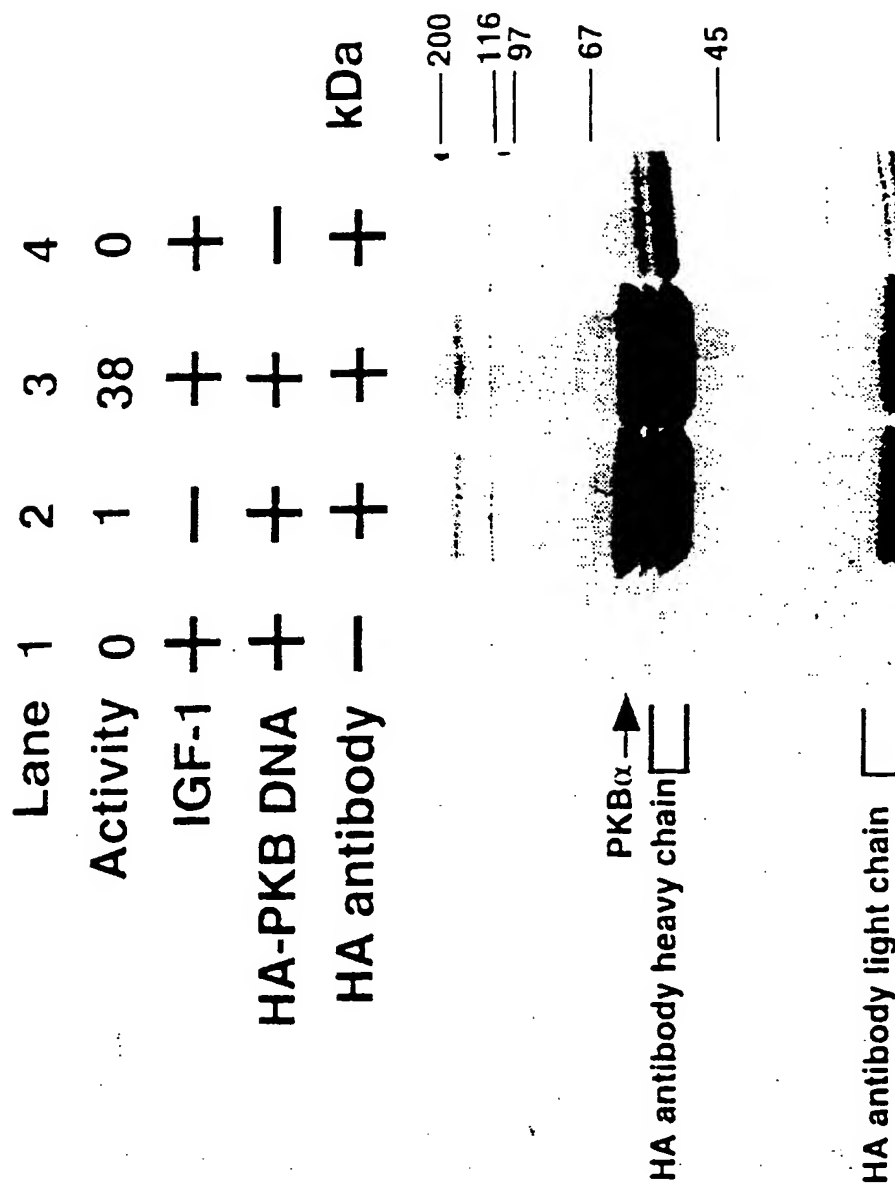


Fig. 16

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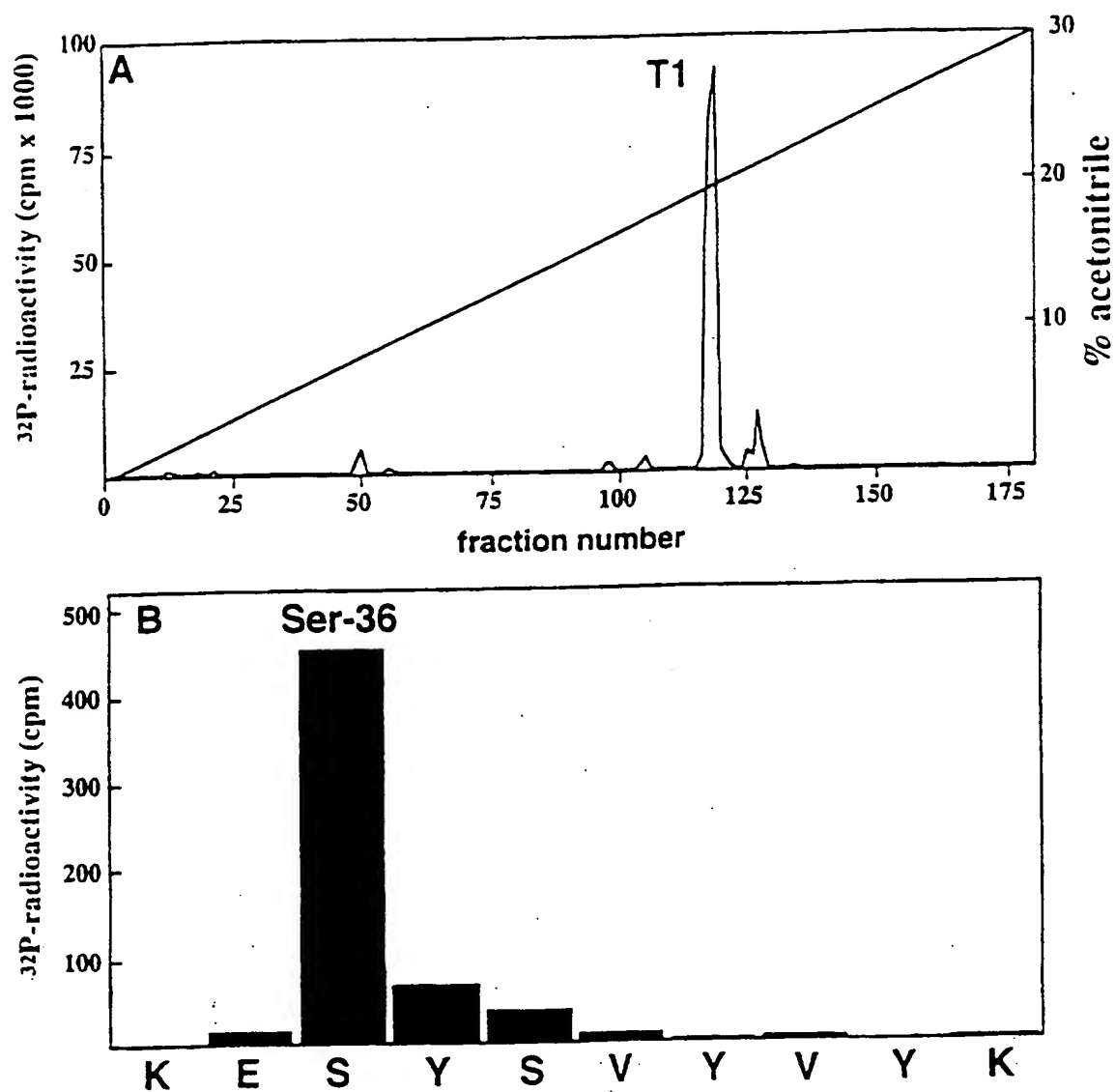


Fig. 17

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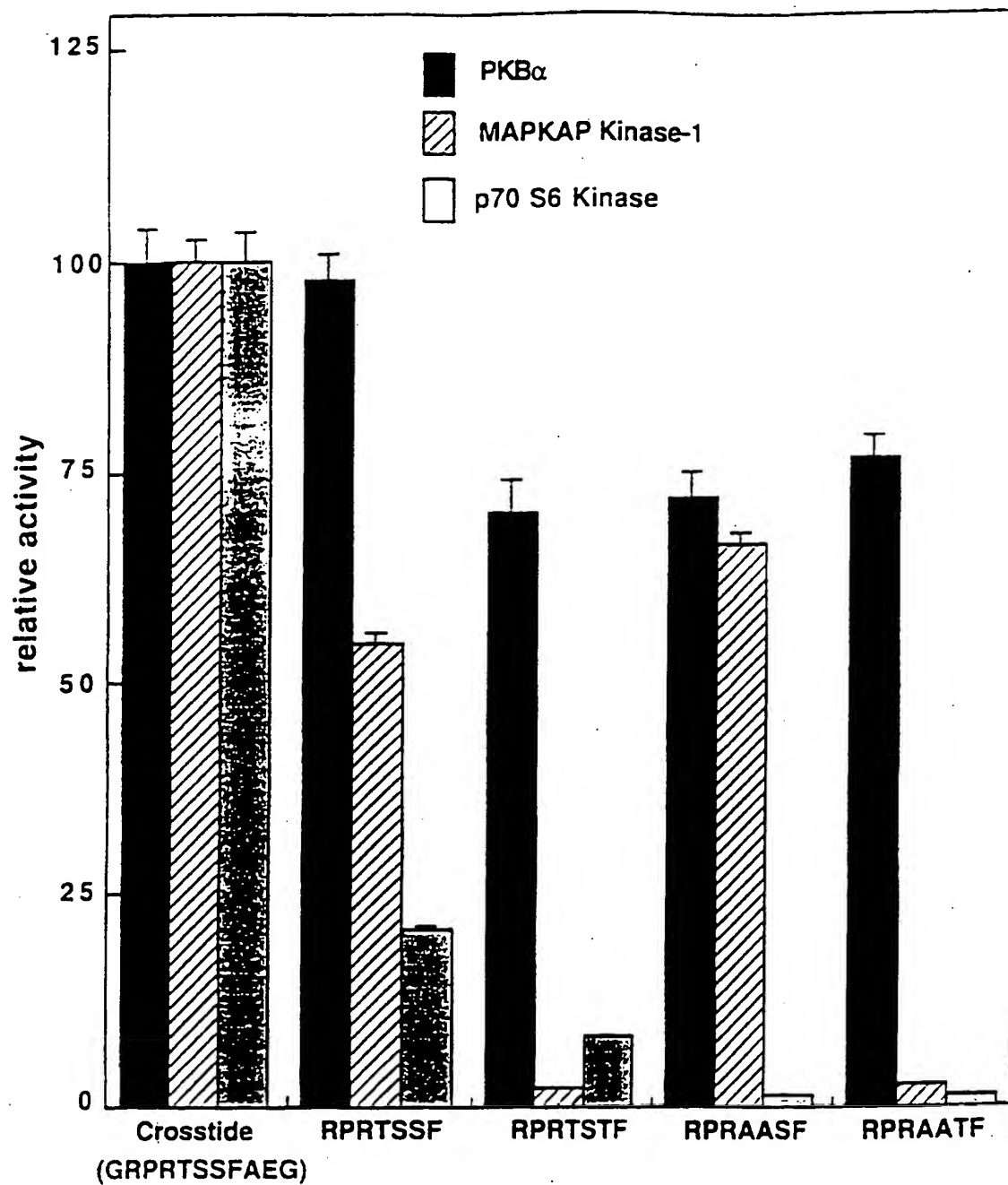


Fig. 18

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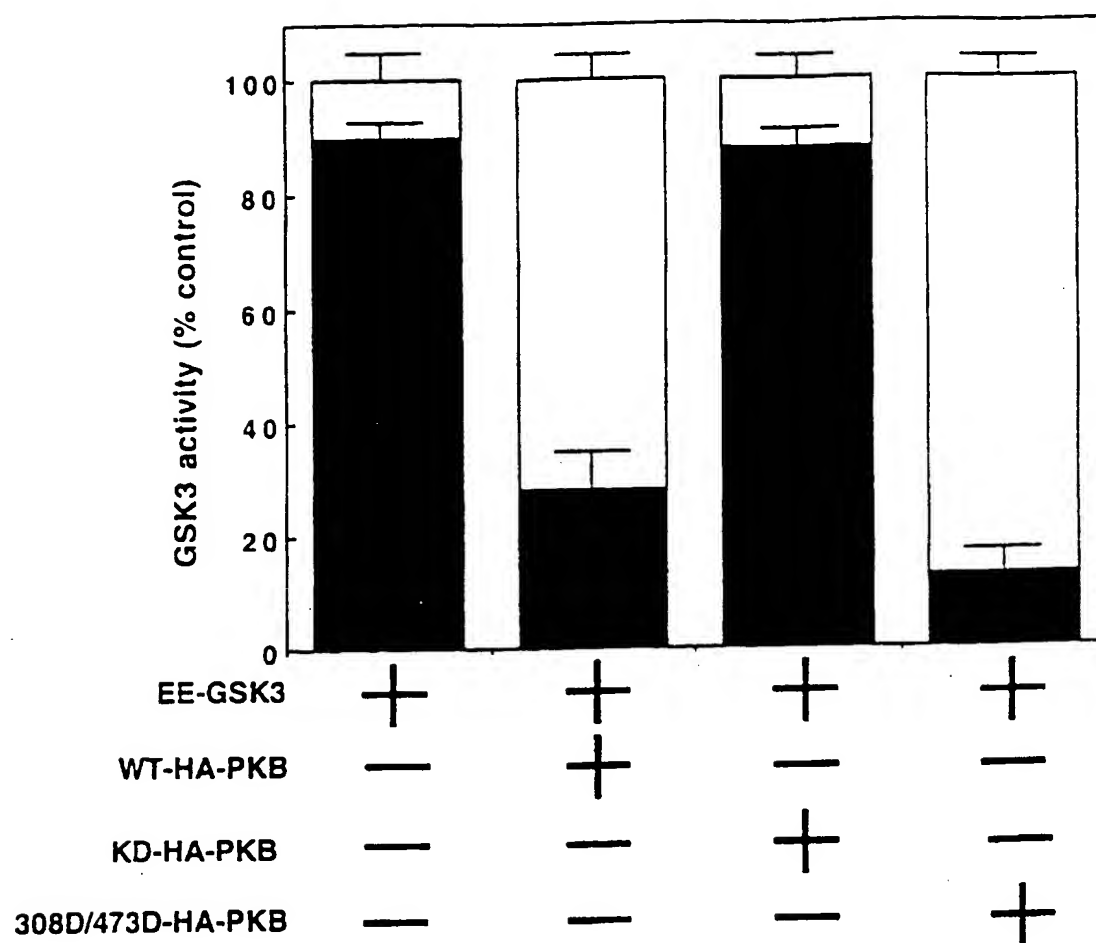


Fig. 19a

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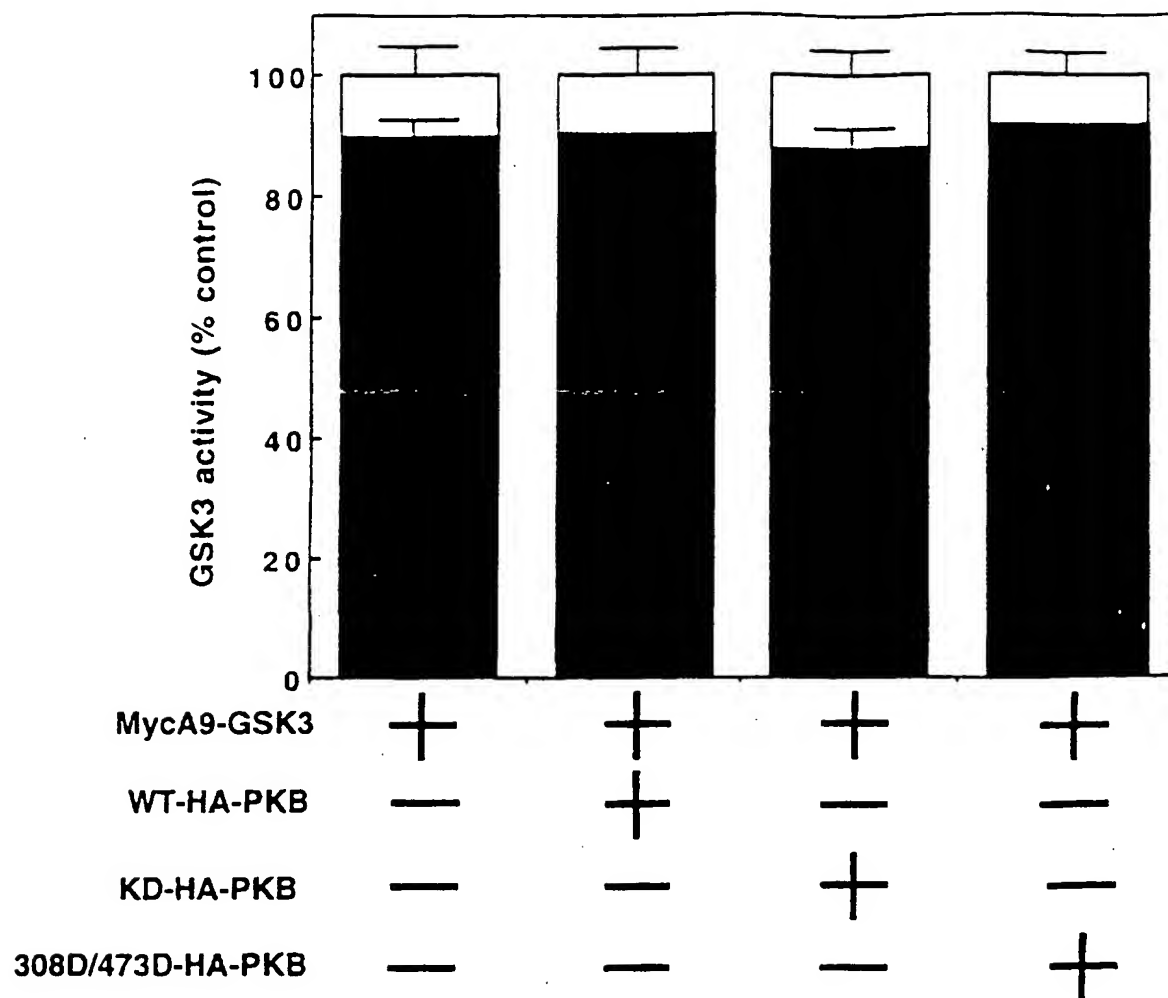


Fig. 19b

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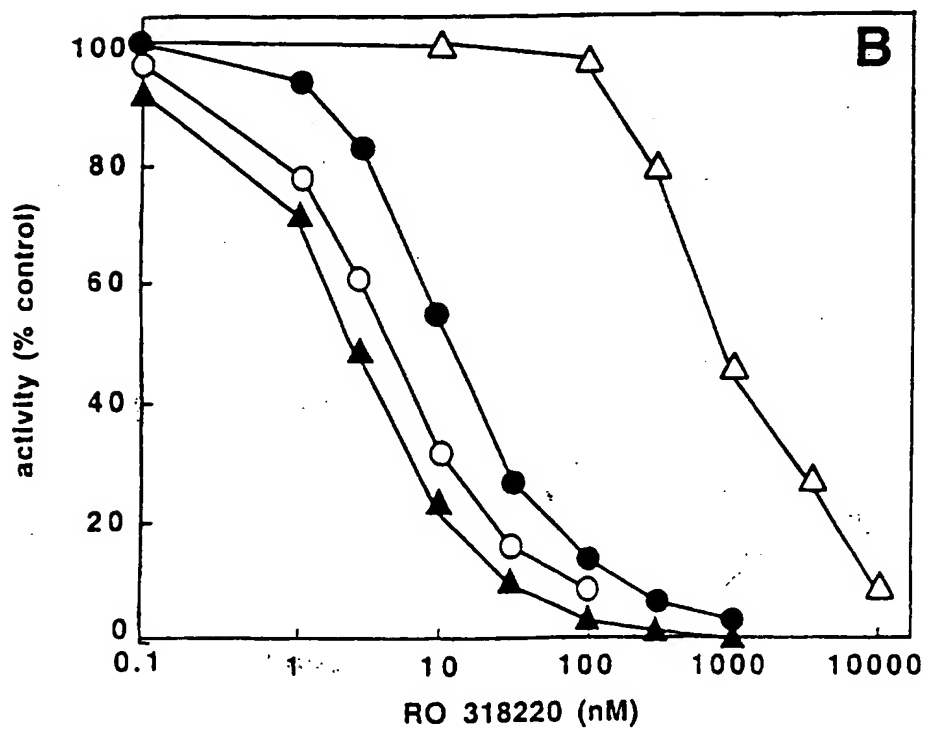
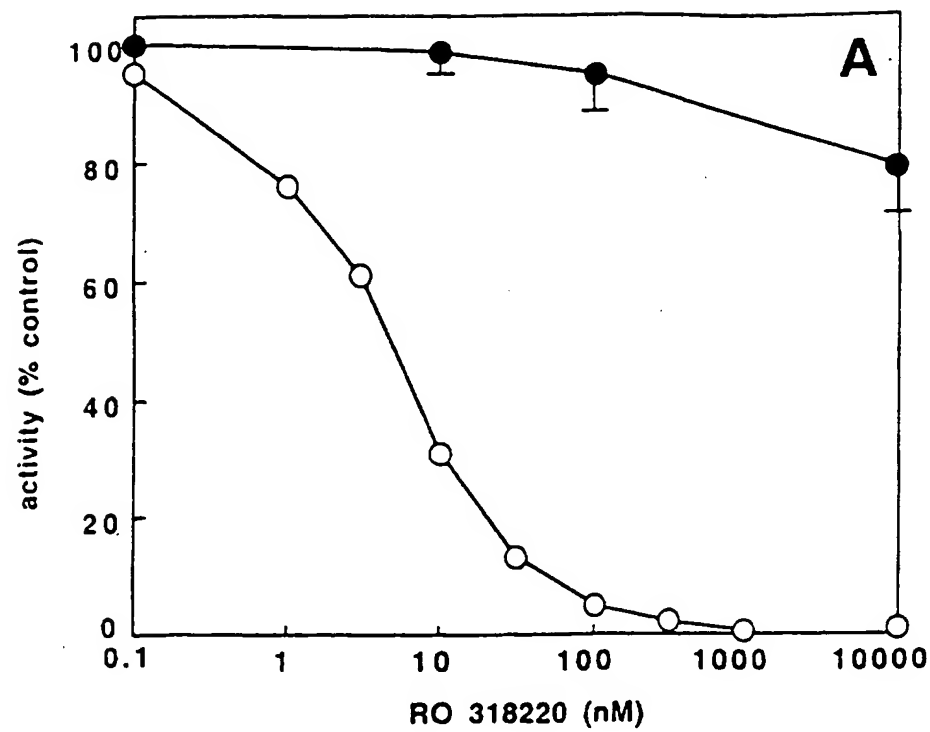


Fig. 20

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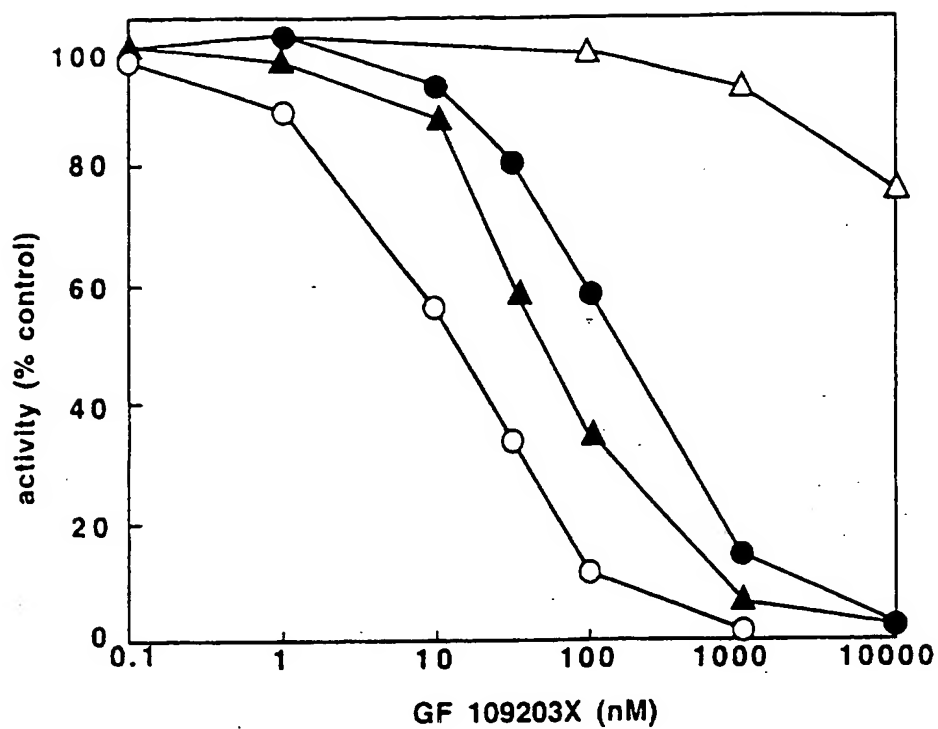


Fig. 21

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